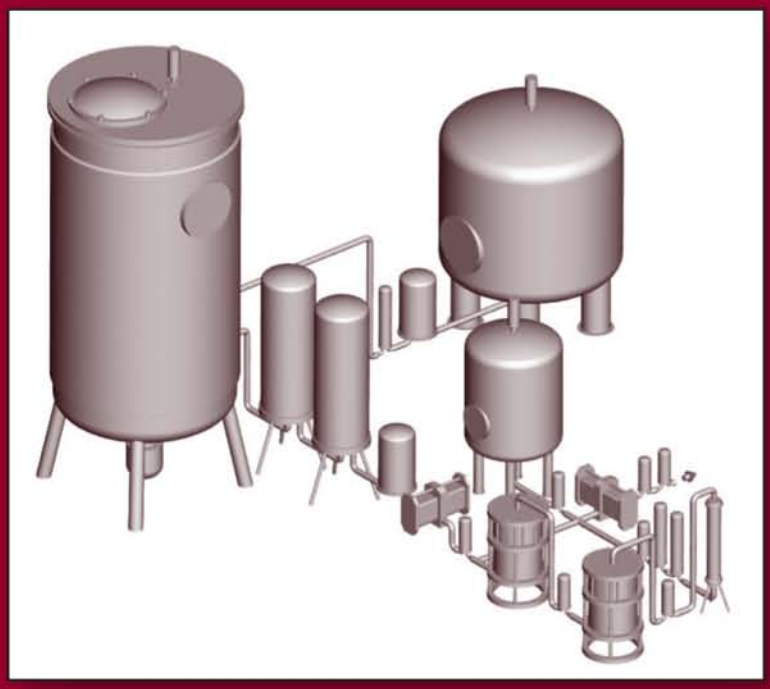
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DRUGS AND THE PHARMACEUTICAL SCIENCES VOLUME 174

Filtration and Purification in the Biopharmaceutical Industry

Second Edition



edited by

Maik W. Jornitz

Theodore H. Meltzer

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Filtration and Purification in the Biopharmaceutical Industry

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Filtration and Purification in the Biopharmaceutical Industry

Second Edition

Edited by

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With love and respect
We dedicate this work to those who inspire and support us

To Xavier Magdalene Meltzer, wife and companion,
To Kathryn Anne Robinson for her steadfast caritas.
— T.H.M.

To Alta Dorette Jornitz, my wife and treasure,
To Lisa Kara Jornitz, my daughter and joy.
— M.W.J.

Foreword

Filtration has been used successfully for microbiological control in water and pharmaceutical manufacture for many years. However, much of the knowledge that was the basis of its use was empirical and incomplete. For many, the idea of filtration was the sieve concept, and the use of terms like "pore size" encouraged that thinking. As a result of this incomplete knowledge, some notable instances of unsuccessful filtration have occurred.

There exists competing pressures for filter selection in the biopharmaceutical industry. In the simplest sense, there is the need to sterilize the solution, which calls for smaller pore size ratings, while conversely there is a need to put the solution through the membrane in a reasonable amount of time, which calls for greater pore size ratings. Understanding the myriad of additional options is a significant challenge that will improve filtration processes.

Understanding the mechanisms of filtration and proper applications of the technique has been the quest of many industrial scientists. The transition from intuitive opinions to scientific conclusions based on physical and biological measurement has permitted a reassessment of our understanding of the mechanisms of filtration. Frances Bowman's early studies probably ignited much of the inquiry relating to this undertaking. However, the technology has remained dynamic, as evidenced by the introduction of new membrane materials, innovative equipment, and new methods to examine filtration. In this revision of the *Filtration* series, up-to-date information is presented on a broad range of issues surrounding biopharmaceutical applications. These topics will assist the reader in developing well-controlled manufacturing processes and preparing quality products.

David Hussong, Ph.D.
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Beltsville, Maryland, U.S.A.

Preface

Since the original edition of *Filtration in the Biopharmaceutical Industry* was published, the industry, technology, and regulations have evolved extensively. Filtration science and technology has made significant advances and is considerably more sophisticated and effective. However, many industry scientists and compliance personnel do not yet hold a thorough and necessary understanding of current filtration capabilities—and pitfalls. This new edition has been created to provide comprehensive, state-of-the-art information on all scientific and technological aspects of filtration.

Filtration equipment in general has evolved considerably in recent years and some products mentioned in the last edition have become obsolete. One key development in the field is that most sterilizing-grade filters have at least doubled their total throughput and flow rates, accelerating filtration processes markedly.

In addition, the manufacturing regulatory environment has become more stringent and validation requirements have changed, increasing focus on the effectiveness of filtration methods and processes. This revised volume reflects these changes and covers the newest FDA and EMEA requirements.

Additionally, the biotechnology industry has matured, requiring that we add technologies which were either non-existent or in very early stages when the last edition was published. As a result, the following subjects have been added to or expanded upon in the new edition:

- Viral retentive filters including important process parameter and validation topics
- Membrane chromatography
- Downstream processing: what technologies are used, which important factors must be taken into account, and how the process differs according to drug targets
- The critical role of purification
- Cell harvesting, which is of great import for biotech-derived products
- Medium and buffer filtration, which are also focused applications, specifically for the biotech industry

ACKNOWLEDGMENT

This book in the third revision of the *Filtration* series, which started as *Filtration in the Pharmaceutical Industry*, was revised to become *Filtration in the Biopharmaceutical Industry*, and is now *Filtration and Purification in the Biopharmaceutical Industry*. As the titles reveal, the industry as well as the technologies have evolved rapidly. Filtration and separation were joined by purification and concentration, as innovative equipment and processes were introduced into the industry. This also meant that previous books

describing essential parts of the drug manufacturing process had to be updated. This venture could only be undertaken by those expert in the particular process steps, or in equipment usage.

The editors of this book would like to express their deepest gratitude to the authors and co-authors of this book. The expertise and experience they contributed to this book was invaluable for the editors, and for the pharmaceutical and biopharmaceutical end-users in search of precious information and support. The contributions of these experts to this book establish an overview of know-how and necessary pointers, important for manufacturing process requirements.

Moreover, we would like to take this opportunity to convey our sincerest appreciation and highest respect to Peter Cooney, Ph.D., who persisted and pioneered sterilizing-grade filter process validation. The definitions and requirements set by him and his team created the benchmark of modern sterilizing-grade filter validation under process conditions using either the actual fluid or a close alternative (placebo).

This benchmark has become a guideline throughout the industry, for filter manufacturers and users alike, and for those dealing with regulatory documentation. It is also seen as a foundation for further validation needs, for example in viral clearance, or for 0.1 micron rated filters. Many of this book's chapters carry forward the concepts and work initiated by Dr. Peter Cooney.

We would also like to recognize Mary Araneo and Sandra Beberman of Informa Healthcare, our publisher, for their inexhaustible patience and support to accomplish this book.

Maik W. Jornitz
Theodore H. Meltzer

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1
Prefiltration in Biopharmaceutical Processes

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PREFILTRATION PRINCIPLES

Prefiltration can be described simply as any filtration step incorporated into a manufacturing process prior to the final filtration. The usual purpose in conducting pharmaceutical filtrations is to remove objectionable particles from a fluid drug preparation. In effecting such a purification there is a concern for the rate at which the filtration takes place, and the extent to which it proceeds before the retained particles block the filter's pores sufficiently to render further filtration so slow as to be impractical. An adequacy of particle removal is the principle goal. The rate of filtration and throughput are secondary considerations. Nevertheless, the accrual of particles on the final filter relative to its porosity and extent of filter surface determines the ongoing rate of filtration as well as its ultimate termination.

In practically all pharmaceutical and biotech processes, the final filter is a microporous membrane, which is manufactured from high tech polymers. It is commercially available in pore size designations of 0.04–8 μm, and due to its mode of manufacture is of a narrow pore size distribution. Consequently, these filters presumably retain particles of sizes larger than their pore size ratings with great reliability,[a] the mechanism of particle retention being sieve retention or size exclusion. Being extremely effective at removing sub-micronic particles, they retain so thoroughly that with heavily loaded liquids they may not have a significant capacity to remove large volumes of particulates while maintaining sufficient fluid flow across the filter. More importantly, the more particulate matter with which the final filter is challenged and retained, the higher the differential pressure across the filter will become. This is undesirable because it is widely known that a filter performs at its highest particle retention efficiency when operated at low differential pressures (Δp). At a low Δp the filter retains small particles through the mechanism of adsorptive sequestration. Lower operating pressure differentials will provide greater throughputs than will high Δp, because the higher pressure differentials tend to compress the filter cakes rendering them less permeable to

[a] This is a popular view of the pore size ratings. It is known, however, that the assigned numerical pore designations are not dimensional measurements. Among other factors, the particle shapes may importantly influence their capture.

liquids. The problem can be solved by the use of larger effective filter areas (EFA). However, this entails the cost of the additional membrane filters. The use of prefilters accomplishes essentially the same purpose, but at a lesser expense.

In reality, therefore, the only reason for prefiltration is based on economic constraints. There are no particulate contaminants in a fluid stream that could not, at least in principle, be removed by the final sterilizing grade filter. However, the cost of filtration would increase significantly under this situation, due to the larger amount of final filtration area that would be required. Prefiltration, by this definition, is a more cost effective means of removing the majority of the contaminants from the fluid stream prior to the final filter removing the remainder. It, therefore, becomes important to incorporate one or more levels of prefiltration so that the particulate challenge to the final filter is minimized, allowing it to operate at the highest level of efficiency.

Prefilters are not intended to be completely retentive (if they were, they would by definition be final filters). Prefilters are designed to accommodate only a portion of the particulate load, permitting the remainder to impinge upon the final filter. In the process, the life of the final filter is prolonged by the use of the prefilter, whose own service life is not unacceptably shortened in the process. Overall, the service life of the prefilter(s)/final filter assembly is extended to the point where the rate of fluid flow and its throughput volume meet practical process requirements.

Depth type filters are usually used for prefiltrations. However, microporous membranes of higher pore size ratings may serve as prefilters for final filters of finer porosities. In such cases it were best, however, that the liquid not be highly loaded, or that more extensive EFA be used to forestall premature filter blockage (Trotter et al., 2002; Jornitz et al., 2004).

CELLULOSE-BASED DEPTH FILTERS

One of the most common prefilters used in biopharmaceutical processes is the cellulose-based depth filter, which is used in either a sheet format or in a lenticular cartridge format. These are very cost effective pre-filters due to the relatively inexpensive raw materials used in their manufacture and the thickness and structure of the filter matrix that is formed during the manufacturing process. The basic raw materials used in the production of these filters are cellulose fibers, inorganic filter aid, and a polymeric wet strength resin (Fig. 1).

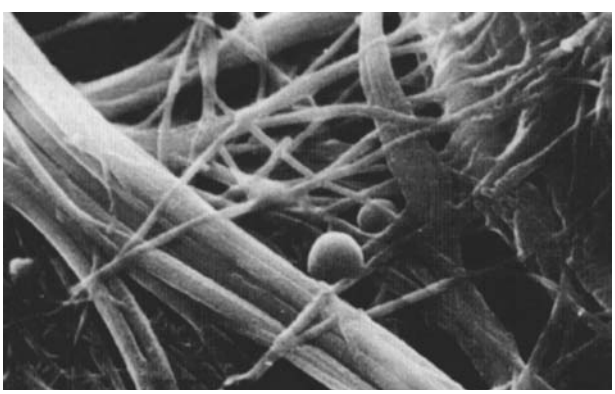


FIGURE 1 Cellulose fibers.

Cellulose pulp is available as either hardwood or softwood. Hardwood is primarily comprised of short fibers which provide a smooth filter sheet surface. However, the short fibers create a dense structure with minimal void volume. Softwood has a longer fiber structure which provides greater void volumes and a mechanically stronger filter sheet with a rough surface.

The void volume of the cellulose and particle retention capacity of the cellulose component of the filter is controlled by refining the cellulose fibers. Pulling the fibers apart creates more and more surface area as the cellulose fibers are fibrillated to a lower freeness. Freeness is a measurement of the ability of water to flow through the fibers. The lower the freeness, the tighter the matrix of cellulose fibers and the smaller the particle retention capacity of the filter media.

The Filter Aid

The next primary component of the filter sheet is the filter aid. The overwhelming majority of filter sheets contain diatomaceous earth (DE) and/or perlite as a filter aid. These two inorganic substances are naturally occurring and mined from deposits in various parts of the world.

Perlite is volcanic ash that has a glass-like, smooth structure. The particles are relatively homogeneous in shape and they form densely within the matrix of cellulose fibers. The powder is available in various particle sizes to provide a range of porosity (Fig. 2).

Diatomaceous earth is comprised of diatoms, which are fossilized remains of plankton. The fresh water variety of this product contains less than 20 different diatoms, while salt water DE is made up of thousands of different species. This broad range of shapes creates a less dense matrix in the cellulose fibers and therefore a greater void volume in the filter sheet. The trade-off is that the variety of shapes creates a filter sheet with less mechanical strength than a sheet made with perlite. Like perlite, DE is available in a variety of grades for various particle retention capabilities (Fig. 3).

The Wet-Strength Resin

The final component of the filter sheet is the wet strength resin. This ingredient serves two purposes; the first is to hold the sheet together and the second is to impart a positive

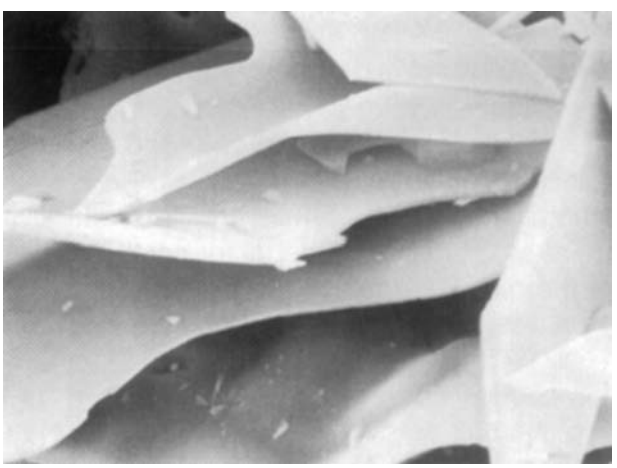


FIGURE 2 Perlite.

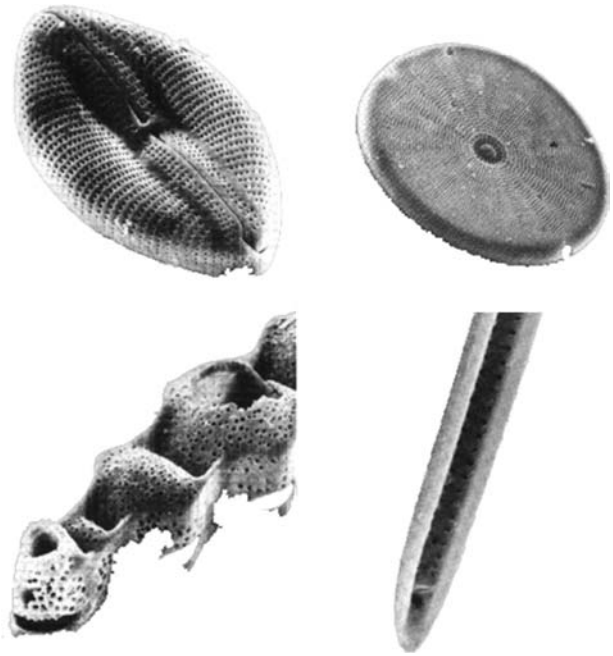


FIGURE 3 Diatoms in diatomaceous earth.

charge to the internal surfaces of the filter sheet. The positive charge is referred to as zeta-potential and it allows the filter to retain particles that are smaller than the pore size by attracting them to these positively charged sites (Fig. 4).

The components can be mixed together in different ratios to affect the performance qualities of the filter sheet, the most important of which are flow capacity and particle retention. Cellulose-based filter sheets can provide effective particle retention down to 0.1 μm. The retention rating of depth filters is stated as a nominal value, essentially meaning that it will remove at least one particle of the stated size. Most manufacturers, however, provide the rating based upon a certain percentage of particles of the stated size under standard conditions. The rating is usually based upon the filter's ability to retain a

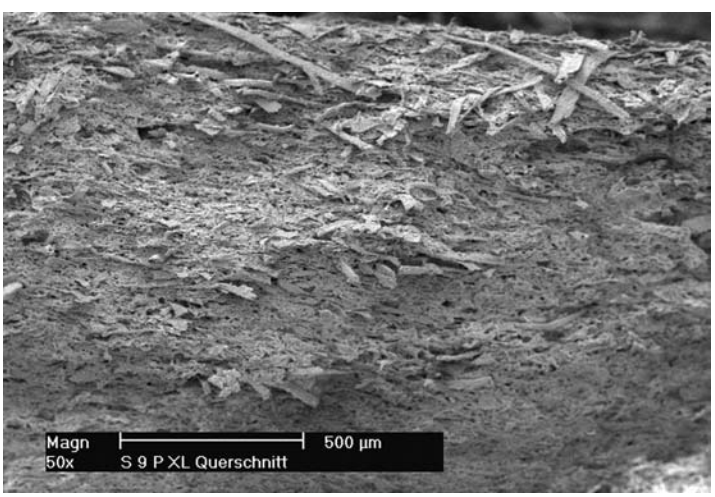


FIGURE 4 Wet strength resin.

fixed-size spherical particle at a constant differential pressure. In practice, most particles are not spherical and filtration studies should be carried out to define the ability of the filter to provide an acceptable filtrate.

RETENTION MECHANISMS

Sieve Retention

There are a number of retention mechanisms employed by depth filters. Among them are: sieving, inertial impaction, Brownian motion, and adsorption.

Sieving is the simplest form of particle retention, and results from the removal of particles that are larger than the pores that they are trying to pass through. The retention is independent of the number of particles or pores, and it is independent of the filtration conditions. For example, unless it is high enough to deform the suspended particle, the differential pressure does not affect the particle removal. The nature (polymeric) of the filter, as also that of the particle, are of no concern unless the physicochemistry of the fluid vehicle reduces the particle size or enlarges the pore size. Each of these is a real possibility under certain conditions (Fig. 5).

The particle may be large enough to be retained at a pore entrance or it may become trapped at a restriction within the pore. If it is deformable; as the differential pressure increases it may become further embedded in the fiber matrix. It is conceivable that if the differential pressure gets high enough the particle could be forced through the pore restriction, deeper into the matrix, and possibly through the matrix back into the fluid stream. Therefore, it is very important to adhere to the pressure limitations as provided by the filter media manufacturer. There are practical limits to the thickness of the filter sheet. The thickness of the filter medium plays an important role in its particulate holding capacity. The thicker the sheet, the more likely the retention. However, depending upon the filter porosity, higher differential pressures may be required in the filtration. Filter manufacturers formulate filter sheets to a thickness sufficient to provide effective filtrations. Sheets that are unnecessarily thick will be too costly to dry following the vacuum formation step of their manufacture. Both economics and practicality are involved.

Inertial Impaction

The inertial impaction of a particle upon a filter surface can occur when the fluid bearing the particle changes its direction of flow as it is deflected into and through the filter pores. The inertia of the particle may continue it on its original path to collide with the filter surface where adsorptive forces can cause its arrest.



FIGURE 5 Sieve retention.

This inertial force depends directly upon the mass of the particle, and the square of its velocity. It is, therefore, more important with heavier particles. The inertial force is attenuated by the viscosity of the fluid, and is consequently influenced by temperature which is inversely related to viscosity. For this reason it is less effective in liquid than in gaseous contexts (Fig. 6).

Brownian Motion

Smaller particles, less heavy, are less influenced by inertia. However, they are more affected by Brownian motion wherein they are vectored from the fluid pathway to the pore surface by collisions with the fluid's molecules. The result is retention of the particles by the filter surfaces they impact.

At all temperatures above absolute zero the various sections of all molecules are in constant motion; the various bonds being flexed, rotated, stretched, etc. The higher the temperature, the greater the amplitude of the molecular motion. The significance of absolute zero is that only at that temperature or below is all molecular movement frozen. In their frenetic activity, the fluid molecules collide, perhaps repeatedly, with suspended particles. The latter are directed to new directions of travel within the fluid stream. As a result of their induced random and erratic movements, the particles have opportunities to encounter pore surfaces. This is the nature of Brownian or diffusional interception. It is favored by small size particles, and by the lower viscosities of the suspending fluids.

Adsorptive Interactions

The donating of electrons by one atom, possibly already part of a molecule, to another one results in a strong bonding between the two atoms. This is the nature of the chemical bond. The same occurs from the sharing of electrons between two atoms. The atomic interactions resulting from electron sharing is called covalent bonding. By convention, electrons each represent a full negative charge. Thus, possessing more electrons than in its neutral state confers a negative charge upon an atom. Having fewer than the normal number of electrons in its possession gives an atom a plus or positive electrical charge.

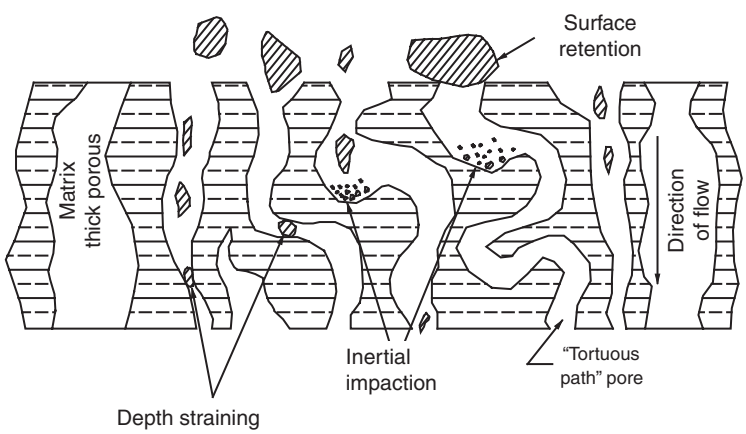


FIGURE 6 Inertial impaction.

Opposite electrical charges attract one another and combine to form a valence or chemical bond. Similar electrical charges repel one another.

It is also possible for atoms, whether individual or as part of a molecular structure, to acquire a partial-electric charge[b] The oppositely partially charged atoms can also combine to form a stable union, albeit weaker than the chemical bond just discussed. This is often referred to as a "physical" bond. Its is similar in its electrical nature, but involving only partial charges it is weaker that the "chemical" bonds derived from full electrical charge interactions. The distinction between chemical and physical is one of degree. The motivating force is identical.

It is the partial-charges that are mostly involved in adsorptions. In the case of particles being adsorptively bonded to filter surfaces, the partial-charge on an atom constituting the particle surface interacts with an oppositely charged atom on the filter surface (pore wall). The result is the adsorption of one surface to the other. The particle adheres to the filter, and in the process is removed from the fluid stream that flows unimpeded through the filter pores.

Adsorption is the bonding of particulates to the surfaces of the pore walls of a filter by any of several electrical charge phenomena. Often referred to as electrokinetic forces, these may be in the nature of strong plus charges resident on a quaternary nitrogen atom that is part of a molecular grouping. This is the case of the depth filters here considered. The adsorptive interaction between the particle surface and the pore surface is accomplished via the electrokinetic charge on the wet strength resin which is used to cross-link the cellulose fibers. Its plus- charge connects with partial-negative charges on the particle surfaces. This enables the removal of particles smaller than the filter pores by bonding them to the oppositely plus-charged nitrogen atoms of the resin molecules that are components of the depth filter surface. In this manner, particles smaller than the pores are rendered immobile by their adsorption to the resin molecules; they are not removed from the fluid stream by sieve retention (Fig. 7).

CHARGE-MODIFIED FILTERS

The resin with its quaternary nitrogen atom is in effect a charge-modified filter. There are, of course, limitations to the use of charge-modified filters. Their performance is stoichiometric in character. Their removal efficiency is strongly dependent upon the encounters between the charged-sites fixed on the filter surface and the oppositely charged particles being carried by the flowing fluid. It is heavily influenced by the rate of flow, particularly as the charged-sites become increasingly occupied. The performance is limited by the finite capacity of the charge entities. Breakthrough becomes increasingly possible as the number of the charge-sites decreases. Thus, where adsorption is dependent on quaternary charge sites, the filter's ability to remove particles through this adsorption mechanism is exhausted when all the fixed-charge sites are occupied. Any additional particles smaller than the pore sizes will pass through the filter and into the filtrate. The use of charge-modified filters is an important technique, but requires careful operations.

[b] Partial-charges derive from several causes: Hydrogen bonding; van der Waal forces; dipoles, both permanent and induced; hydrophobic adsorptions; etc. The various influences, for example, zeta potential, ionic strengths, Debye lengths, etc. that are operative in the double electric layer phenomenon may be involved (see Meltzer and Jornitz, 2006 and references therein).

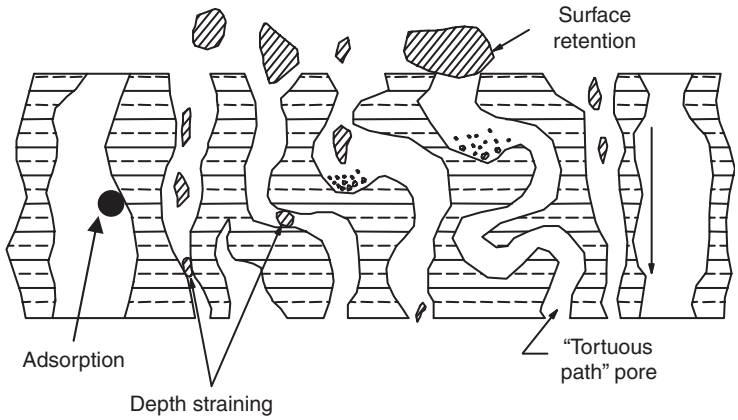


FIGURE 7 Adsorptive interactions.

Activated Carbon

Activated carbon presents an enormous extent of surface for the adsorptive interactions leading to the removal of many different types of impurities. Activated carbon is prepared by the thermal degradation of carboniferous materials in an atmosphere limited in its oxygen content. It has strong adsorptive capacities. This is a consequence of its enormous surface area when in finely divided form. Additionally, the thermal decomposition in a restricted oxygen atmosphere introduces certain oxygenated molecules onto the carbon's surface that are conducive to adsorptions. This furnishes an impressive extent of surface area for adsorptive interactions leading to the removal of many different types of impurities (Fig. 8).

As such, activated carbon is commonly used for the removal of metal catalysts in conjunction with a horizontal plate filter. It serves as well for the removal of organic substances, and of color-bearing (chromophoric) molecules.

The use of finely divided activated carbon risks its undesired dissipation throughout a filtration system. Its fine size makes easy its distribution by the flowing fluid. Its use requires careful management. One option is to utilize filter sheets containing activated carbon used as an ingredient in the formulation of the filter medium. This can eliminate the use of loose powdered carbon, but the lower amounts present in the filter sheets may offer a more restricted capacity.

Filter Forms

Cellulose-based depth filters are available in two configurations, flat sheets and lenticular cartridges. These two configurations are used in plate and frame filter presses and filter housings respectively. Plate and frame filters have been used for over 100 years and

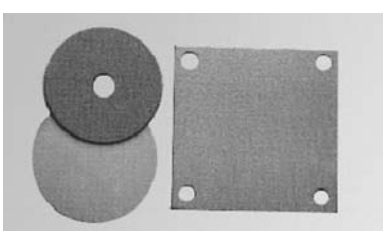


FIGURE 8 Activated carbon filters.

provide the ability to build and wash filter cakes. They utilize flat sheets which are less expensive than lenticular cartridges by a factor of approximately 5:1 based on cost per unit area of filtration. The plate and frame filter requires the opening and closing of the filter in order to load and unload the filter sheets, and this is time consuming and the design tends to leak. Plate and frame filters are available in either an internally ported or externally ported design (Fig. 9).

With an externally ported filter press the filter sheet has no holes and the flow channels are external to the filter plate dimension. This requires the use of eyelet gaskets to seal the flow port channels. The individual sheets must also be sealed between the plates and frames which means that there are five independent sealing surfaces between each plate (Fig. 10).

Internally ported filter presses have holes in the filter sheets and the flow channels are internal of the plate and frame. This design will have less active filtration area than an externally ported filter with the same plate dimension. However, due to the internal ports, only the filter sheet is required to be sealed between each plate and frame (Fig. 11).

Filter presses are commonly used in the Cohn fractionation process to collect precipitated paste, which is subsequently washed with a solvent to resolubilize the precipitate for further processing. Plate and frame filters are also used in the filtration of oral syrups and other pharmaceutical preparations that are manufactured in high volumes and have relatively low value per unit volume.

Another type of plate filter is the horizontal plate filter which can use filter paper or filter sheets, and is commonly used for catalyst recovery in active pharmaceutical ingredient (API) applications.

Use of the horizontal plate filter is even more labor intensive than the open vertical plate and frame filter. It consists of a plate stack that is assembled outside of a vessel and held together with tie-rods that are tightened around the outside diameter of the stack. The assembled unit must then be hoisted in the air and lowered into the vessel where it is secured in place. The vessel is closed and the filtration process can commence (Fig. 12).

Lenticular Configuration

The lenticular configuration was introduced in the 1940s by Alsop Engineering, and was specifically designed to provide an enclosed version of the plate and frame filter that was

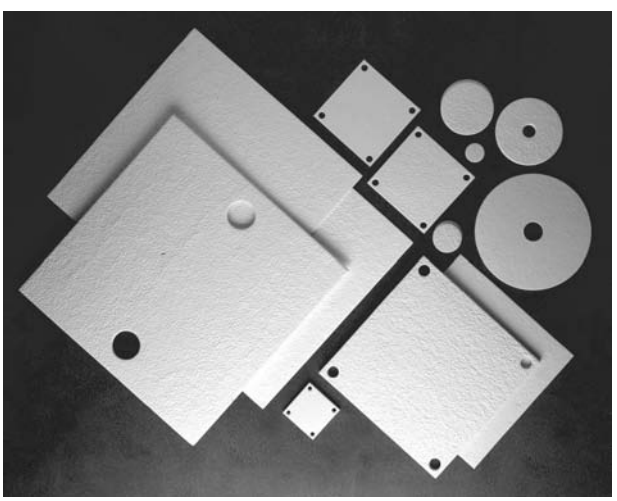


FIGURE 9 Plate and frame filters.

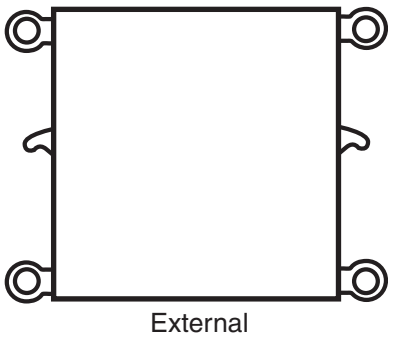


FIGURE 10 Externally ported filter sheet.

easier to change than a horizontal plate filter. Lenticular filter cartridges are available in three nominal diameters, 8-, 12-, and 16-inch. The cartridges are made up of a series of cells that are stacked on top of one another with plastic rings between them to prevent bypass between the cells. End-caps are then placed at the top and bottom of the assembly and held in place with either stainless steel straps or a polypropylene core. The end-caps can be provided to accept either flat gaskets or dual o-rings (Fig. 13).

The filter housings that these filters are placed in are available in vertical or horizontal orientations and can hold up to twenty cartridges. The two primary advantages of this design are the enclosed aspect of the operation, and the ease of filter replacement between batches. This makes the lenticular design very popular in the filtration of many pharmaceutical and biotech products (Fig. 14).

A recent development in fermentation processing is the use of disposable filter devices in the several steps from the bioreactor through the final fill. Disposable depth filters are available for use in these systems.

FIBROUS MATERIALS

Glass Fibers

The type of filtration applications utilizing depth filters is large and varied. Use is made of fibers of many compositions in addition to the cellulosic prefilters. Glass fibers find application in serum filtrations. These include borosilicate glass fibers, some with special coatings such as of nitrocellulose polymers. These coatings are very effective in the removal by adsorption of process impurities, especially of protein-like matter, and of

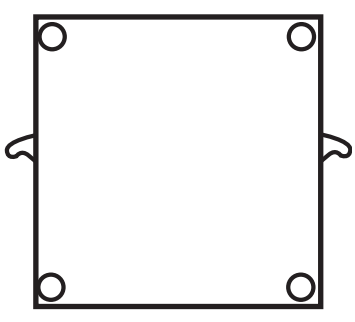


FIGURE 11 Internally ported filter sheet.

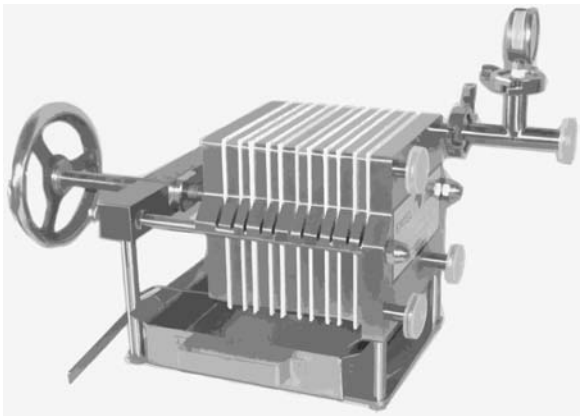


FIGURE 12 Horizontal plate and frame filter.

lipids. The nitrated polymer likely performs its adsorptive removal of impurities through the agency of hydrogen bonding.

Polypropylene

Polypropylene fibers are widely used. They can be formed into fleeces and mats of various fiber diameters that are bonded together and are permanently cross-linked by heat, being joined by melting, to minimize or eliminate media migration. The melt-spinning method of permanently fixing the fibers to one another, satisfactory in its own right, replaced the adhesives, and mechanical manipulations of earlier and less effective methods of mat formation. In turn, the melt-blown technique now supersedes even this fabrication of polypropylene fleeces by varying the mean fiber size during the fashioning of the prefilter. This enables the progressive changing of the mat's pore size while it is being constructed. The result is a highly graded asymmetric composition having a constant packing density. One advantage is that, in effect, a series of prefilters is combined into the making of the single prefilter composite. The asymmetric morphology provides less resistance to flows, requires lower differential pressures in their operations, provides for the accommodation of higher particle loadings, and offers greater particle removal due to the efficiency of thinner fibers. Fibers of diameters as small as 0.3 μm in their mean size provide the retention of particles from a fluid stream down to 1 μm in size.



FIGURE 13 Lenticular filter cartridges.



FIGURE 14 Lenticular filter housing.

The graded pore size format is derived by decreasing the fiber diameters rather than by increasing the mat's density by tighter packing. This enhances the prefilter porosity, and provides the additional advantages of lower operational differential pressures, and larger load accommodations.

EXAMPLES OF APPLICATIONS

The use of prefilters is applied to numerous applications; indeed, almost universally in process filtrations, so helpful is the beneficial effect of prefiltration. Following are some examples of applications wherein prefiltration plays an important role.

Active Pharmaceutical Ingredients

Depth filters are primarily utilized in two locations in API processes, namely, catalyst removal, and as prefilters to final membrane filtration. In many API applications activated carbon is used to remove metal catalysts. The activated carbon can be added as a loose powder retained on the surface of a filter paper or depth filter sheet. Or the activated carbon can be included in the depth filter formulation to eliminate the use of the loose powder. In this application, contact time with the carbon is the most critical factor in achieving a successful filtration. This can be accomplished by maintaining low flow rates per unit filtration area and low differential pressures across the filter. The activated carbon adsorbs the metal ions remaining after the catalysis. The catalyst and its value is recovered after the process is complete.

Another application into which depth filters are incorporated into a filtration step is prior to crystallization of the API. In this phase of the process the depth filter is used as a prefilter to a membrane filter. Its primary purpose is to protect the membrane filter from fouling during the processing of the entire batch. This is usually performed using a lenticular cartridge configuration upstream of the membrane (Fig. 15).

Blood/Plasma Products

Filtrations perform a very necessary function in the preparation of therapeutic blood proteins. The practice has a three-part purpose, namely, to clarify the filtrate, to remove particulates in the prefiltration stage that might otherwise block the final filter, and to sterilize filtratively the treated preparation.[c]

Let us first consider the need for clarification. An example of such a requirement occurs at the end of the Fraction IV centrifugation; the precipitate is found not to be removed with complete efficiency. The supernatant solution does contain suspended matter; nephelometry is used to determine the extent. Filtration with depth-type filters is resorted to in order to achieve clarity.

The depth-type filters most commonly used are the positive charge-induced prefilters, those having positive zeta potentials; mixed esters of cellulose-coated paper prefilters; and coarse and/or fine glass fiber layers, in conjunction with a 0.8-μm-rated microporous membrane consisting of a vinyl acrylate copolymer coating deposited on a nylon or polyester mat support. Filter aids, in particular DE, are often used in conjunction with the prefilter treatment to increase the dirt-holding capacity of the filter system.

PRETREATMENT AND PREFILTRATION

In large-volume parenteral filtrations prefilter action is often the key to successful filter operations. This is particularly true where heavily laden liquids, such as serum and plasma, are concentrated. The particulate load is best reduced by pretreatments involving adsorbents, such as DE, or finely divided fumed silica, followed by depth-type

[c] The usage of blood and serum for the preparation of therapeutic agents is too extensive a subject for this writing. However, the separation and purification of certain components of these fluids are here selected as applicational examples of the utility of prefilters, and filter aids in the production of biopharmaceuticals.

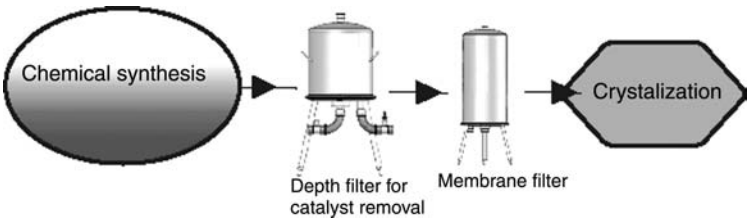


FIGURE 15 Depth and membrane filters incorporated into a filtration step prior to crystallization of the Active Pharmaceutical Ingredients.

prefiltration, before the liquid is permitted to encounter the final, sterilizing microporous membrane filter. Otherwise, premature clogging of that filter will occur.

The purpose of the pretreatments is to present large surface areas on which adsorption and impurity retention may occur. Hence, the use of fumed silica, a finely divided particulate material especially suited for removing lipids and lipoproteins (Condie and Toledo-Perreyra, 1976). The depth-type prefilters likewise offer large surface areas for impurity depositions, e.g., fiberglass prefilters. Additionally, however, prefilters consisting of mixed esters of cellulose coatings on paper (cellulose fiber mats) promote purifying adsorptive sequestrations through hydrogen bonding to their nitro group moiety, as stated above. Positive charge modified depth filters are especially suitable as prefilters in serum/plasma prefiltration. They offer positive-charged sites for particle capture, as well as large surface areas for particle adsorption. In consequence, asbestos filters, previously used extensively, are now at least partially successfully substituted for with success by the charge modified depth filters (Holst et al., 1978).

Pretreatment Agents

Fiore et al. (1980) describe successful applications of pretreatments and prefiltrations relative to therapeutic blood protein preparation. Fumed silica and DE are used as pretreatment aids. The fumed silica (degussa aerosil) has a surface area of 200 m^2/g, diatomite a surface area of approximately 2 m^2/g. Condie and Toledo-Pereyra (1976) advise that lipoproteins, triglycerides, cholesterol, fibrinogen, and the plasminogen-plasmin system present in 1 l of plasma are adsorbed onto 40 g of fumed silica within a contact time of 60 min. This is significant, because Olson and Faith (1978) report that lipoproteins serve to clog microporous filters. Similarly, fibrinogen has a clogging action on such membranes, particularly as it may form fibrous deposits of fibrin in the operation of the blood-clotting mechanism. The adsorptive preremoval of these substances renders more practicable subsequent filtration of plasma/serum. Diatomite has long been utilized in the pretreatment of plasma (Cohn et al., 1946). Depending on how heavily laden the plasma/serum is, how difficult it is to filter, from 2.5 to 5 g are used per liter of the liquid. Removal of the silica or diatomite can be effected in several ways, such as by filtration utilizing prefilters of the type herein discussed. The efficiency of such "filter aid" pretreatments is judged by the clarity of the plasma/serum filtrate as determined by nephelometry (Cohn et al., 1946; Holst et al., 1978).

Prefliters for Plasma/Serum

As stated in Chapter 2, the development of positive charge modified prefilters permitted the replacement of asbestos filters for the prefiltrative purification of plasma/protein, the

handling of asbestos having become interdicted by the EPA on account of the carcinogenicity of certain of its manifestations.

Cohn Fraction IV4 supernatant is a material that usually requires clarifications not easily achieved. Sheet filtration has often been relied on for this purpose. According to Fiore et al. (1980), Seitz 1-μm sheets in a plate-and-frame arrangement, 2 ft^2 of surface per 40–45 liters of supernatant IV4, do the job. Fiore et al. also list Ertel, Cellulo, and Alsop filter media as being essentially equivalent. In a typical operation, the plate-and-frame arrangement is prerinsed with 40% aqueous ethanol solution at −5˚C; some 5 l/ft^2 of filter pad is used. Filtration of the supernatant fraction IV4 is then commenced at a differential pressure of 5–7 psi or less. DE in the amount of 2.5–5 g/l of supernatant fraction IV4 may be added to the rinse solution to form a precoat on the pads. Also, a similar amount of diatomite may be stirred into the supernatant fraction IV4 itself. Since such filter pad constructions inevitably exhibit filter medium migration, the filtrate that issues initially is re-filtered, such as by recycling, to have the fibers that were set loose in the filtration removed by the pad filter action. The temperature during this filtration is kept at –5˚C.

Substitution of a positive charge modified prefilter, such as one composed of cellulose and diatomite in the charge modifed form, is comparably effective to asbestos in its clarifying action (Holst et al., 1978). The charge modifed prefilter is prerinsed with WfI to which 2.5–5 g of diatomite is added per liter of supernatant fraction IV4 to be filtered. (A diatomaceous precoat is thus formed on the prefilter surface.). Some 7 l of prerinse is used per square foot of prefilter surface. To the fraction IV4 supernatant is then added 2.5 l of diatomite, and filtration, using a bell housing is, performed at an applied differential pressure of approximately 5–7 psi at –5˚C. About 1,000 l can be filtered through a prefilter cartridge of 11 ft^2 of effective filtration area.

Holst et al. (1978) report that using suitable pore-size rated positive charge modified prefilters supernatant fraction IV1, ordinarily difficult to clarify, can be treated effectively, 40 liters being processed per square foot of filter. Similarly, Fraction IV4 and Fraction V paste, reconstituted to a strength of 5% protein after lyophilization to remove residual alcohol, and subsequent to certain processing steps, can be filtered at –5˚C and pH 7.1 to an extent of 15 liters/ft^2 of filter surface at an applied differential pressure of about 10 psi. The clarity of the filtrate is thus improved to 10 nephelometric units from 40 in the unfiltered feedstock.

Prefilters of fiberglass, are reported to be effective for plasma/serum, particularly so for the removal of denatured proteins, and lipoproteins, especially of the beta and pre-beta varieties. For this reason, pleated cartridge constructions of a course glass fiber upstream of a fine glass fiber are widely used as plasma/serum prefilters.

Serum Filtration

In one example of serum filtration, the serum is first treated prefiltratively with DE. A cake is made of the diatomite in a 273-mm Buchner funnel. The formation of this filter cake is an artful practice. If the cake is too tightly packed, the rate of plasma flow may be too slow, and the throughput may become abbreviated by premature clogging of the small pores of the compacted cake. If the cake is too open, insufficient adsorptive purification of the serum may result. Such a "Jello®" cake is undesirable. It yields a cloudy serum filtrate where clarity is being sought. In accordance with adsorptive purifications, the thicker the diatomite cake, the greater the product clarity, but also the larger the loss of expensive serum by inclusion within the cake.

The proper cake formation of the DE slurry, and its mode of distribution within the Buchner funnel are all important. The successful exercise of building a cake of the desired consistency is one based largely on experience. Its purpose is to remove lipids, triglycerides, lipoprotein, and cholesterol from the serum.

The temperature of the filtration is important. The serum is usually maintained at 2–8°C, but may be kept at room temperature during filtration, depending on the concerns regarding organism growth. The rate of filtration is about 18 liters over a 20-min period.

Following treatment with the filter aid, the clarified serum is prefiltered through successive disks of extra thick fiberglass, namely, 2- and 1.2-μm-rated fiberglass. Membrane filtration is then undertaken using 0.8- and 0.45-μm-rated membrane filters in series. The first 10 liters of effluent serum are recycled.

The filtered serum usually contains fewer than 100 organisms per milliliter, as determined by the pour-plate technique using blood agar. The organisms of concern are the *Escherichia coli*, *Pseudomonas*, and possibly other in-plant flora. In order to stabilize the filtered serum, proprietary preservatives, such as sodium azide, and broad-range antibiotics are added. Lyophilization of the serum serves to increase its shelf life.

Plate-and-Frame Filtration of Serum

Plate-and-frame assemblies utilizing cellulose pads have been used to remove the lipid and lipoprotein components prior to membrane filtration. A 20-plate assembly has sufficed to filter some 200 liters of serum; 10 liters of serum being factored per plate. Prior to the introduction of the serum, the plate assembly is prewashed with saline, sodium citrate solution, or whatever liquid is appropriate to that particular serum application. An air blow-down of the press to eliminate as much as possible of the washing solution follows. Serum filtration is then commenced. The first quantities of serum collected are recycled to minimize the diluting effect of the remainder of the prewash solution.

Plasma Fractionation

Blood Plasma can be separated into several components for therapeutic products. Many plasma processors use the Cohn Fractionation process to separate the various components. This procedure selectively precipitates out certain components while allowing others to stay in solution. The residual “paste”, which is typically retained in a plate and frame filter press fitted with filter paper, contains the precipitated fractions. The precipitates are then re-solubilized with a washing procedure and recollected for further processing. This method is used on each of the plasma fractions in a similar fashion. Depth filters are also used as pre-filters to sterilizing grade filters prior to the final fill. Once again the depth filter is used to remove the majority of the remaining particulate to protect the sterilizing filter and prevent it from fouling (Fig. 16).

Cohn Fractionation Procedure

Human plasma is collected and is immediately frozen to minimize compositional alterations and bacterial growth. Testing of the collected plasma is conducted to ensure its freedom from such infections as hepatitis, syphilis, etc. The variously collected plasma is then thawed and pooled in a holding tank at 4 ± 1°C. A crypoprecipitate is formed and is

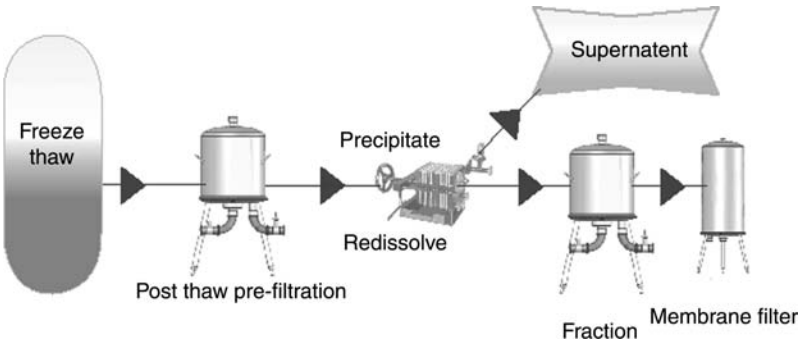


FIGURE 16 Plasma fractionation.

separated by centrifugation. It contains the antihemophilic factor, fibronectin (cold-insoluble globulins), and fibrinogen.

The cryo-poor plasma pool is then adjusted with regard to pH, and cold ethanol is added to a specific concentration. The pH is usually 7.4 ± 0.2, the alcohol concentration 8%, and the temperature $-2 \pm 0.5^{\circ}C$. This treatment yields a fraction I precipitate, and a supernatant I.

Treatment of supernatant I with 20% ethanol at pH 6.8–6.9 at $-5 \pm 1.0^{\circ}C$ yields fractions II and III precipitate plus supernatants II and III. Adjustment of the pH of supernatant to 5.2 ± 0.1 at $-\pm 1^{\circ}C$ results in fraction IV precipitate. If treated at pH 5.9 ± 0.05 at the same temperature with ethanol at 40%, the fraction IV4 followed by pH adjustment to 4.6–4.8 at $-\pm 0^{\circ}C$ and the addition of ethyl alcohol at 40% yields fraction V as a precipitate along with the supernatant V.

Another common procedure for obtaining the plasma protein fractions is to combine fractions I, II, and III at 20% ethanol, fractions IV1 and IV4 at 40% alcohol, and fraction V at 40% alcohol. The point being made is that there are variations of the original Cohn fractionation that are practiced.

Albumin

In essence, fraction V is crude human albumin. It contains salts and residual alcohol. It is dissolved in distilled water, and made up to 10% ethanol concentration at pH 4.6–4.8 at $-3 \pm 1^{\circ}C$. It is then passed through a depth filter to remove any extraneous, insoluble proteins. The filtered solution is readjusted with alcohol to 40% strength at pH $5.2 + 0.1$ at $-6 \pm 1^{\circ}C$. A supernatant is removed by centrifugation to leave a precipitate of albumin.

The reworked albumin precipitate is dissolved in distilled water, prefiltered through 3–8 μm-rated depth filters, and final filtered through 0.2-μm-rated membranes. The albumin solution is then concentrated, usually by one of three methods, namely, lyophilization followed by reconstitution with sterile water, by thin-film evaporation, or by ultrafiltration. Where lyophilization and reconstitution are utilized, the resulting nonsterile bulk solution is stabilized and again filtratively sterilized by passage through depth and final filters into a final sterile bulk tank. Where thin-film evaporation is used, the resulting fraction V precipitate is dissolved in distilled water, stabilized, and clarified by use of prefilters before being subjected to the thin-film evaporator. Following this, the concentrated solution is again prefiltered and final filtered through a sterilizing membrane. Where ultrafiltration is employed as the means of concentration, the procedure is the same except that ultrafiltration is substituted for the thin-film evaporator.

The sterile bulk albumin preparations are assayed for suitability in terms of their protein concentrations, sterility, non-pyrogenicity, pH, purity, turbidity, and safety; after which they are sterile-filled into their final containers subject to QC release and submission to the Bureau of Biologics.

In one operation wherein albumin is prepared in 5% and 25% strengths, some 7001 of the preparation is sterilized by filtration through a steam autoclaved, 0.2-μm-rated membrane filter at an applied differential pressure of 20–25 psi maximum. Cotton plugs are used as the vent filters on the tanks.

Factor 9

AHP-poor plasma at 3–9°C is freed of sodium and other salts, for example, by electrodialysis. The pH is adjusted to just below neutral and the temperature is maintained at 5 ± 2°C. An ion-exchange resin is mixed into the plasma to bind the coagulation factors II, VII, IX, and X. The resin with its adsorbed coagulation factors is separated in a basket-type centrifuge. The resin is then suspended and washed with buffer of pH 6.7 ± 0.1 and placed into a column. Elution is then made from the ion exchanger using cold buffer. The eluted fractions containing the coagulation factors are filtered through a 0.2-μm-rated filter and bottled for storage in frozen condition. Concentration of the frozen factor 9 solution can be had by permitting its thawing to 3–5°C followed by ultrafiltration. Its pH is adjusted to 6.5–7.0, and it is prefiltered through mixed esters of cellulose-coated paper prior to its filtrative sterilization through a 0.2-μm-rated membrane to yield the sterile bulk solution of factor 9.

Oral Syrups

In the filtration of oral syrups, the depth filter is used to clarify the solution prior to filling and is generally followed by a cartridge filter to protect from any particulate that might pass through the depth filter (Fig. 17).

Fermentation Solutions

Particulate from bioreactor offloads can be significant and broad in particle size. The particle size is largely a function of the cell type used in the fermentation process. For instance with mammalian cells, there is a component of the cell debris that is 6–12 μm in size and another in the 0.5–2 μm range. This creates a need for multiple stages of separation. In some instances a centrifuge will be placed at the outlet of the bioreactor, followed by a depth filter to remove the remainder of the particulate for protection of the

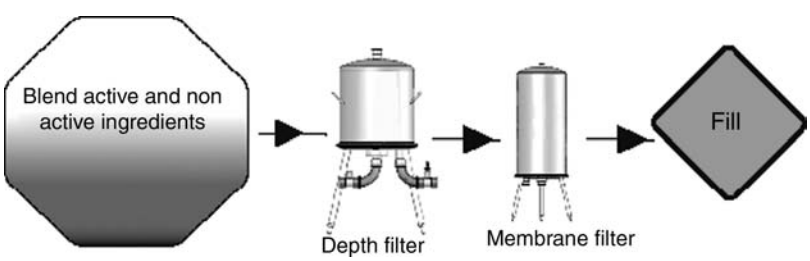


FIGURE 17 Oral syrup filter system.

chromatography stage. The other option is to use two stages of depth filters to effectively remove the wide range of particle sizes.

Depth filters can also reduce virus levels in process streams and are very effective in prolonging the life of virus removal membrane filters (Fig. 18).

FILTER SELECTION

When selecting an appropriate prefilter, the following characteristics should be considered:

1. the ability to protect downstream processes and final filters;
2. chemical compatibility of the filter;
3. thermal characteristics of the process.

Cellulose-based depth filter sheets are capable of withstanding temperatures in excess of 300°F (150°C). However, when configured in a lenticular cartridge the polypropylene components limit the thermal stability of the filter to that of the polypropylene or other polymer that may be used.

A filter that is chemically compatible with the process fluid is one that will maintain its integrity and structure under the process conditions of the application. Most filter manufacturers provide chemical compatibility information, but trials should always be performed to assure suitability of the filter in the fluid to be filtered. Tests to make this determination could be a soak test for an extended period of time at the process temperature or a forward flow test with the unfiltered fluid under conditions that are the same as the process. Following a soak test the mechanical strength of the filter can be tested, or it can be placed in a filter holder and tested for particle retention efficiency.

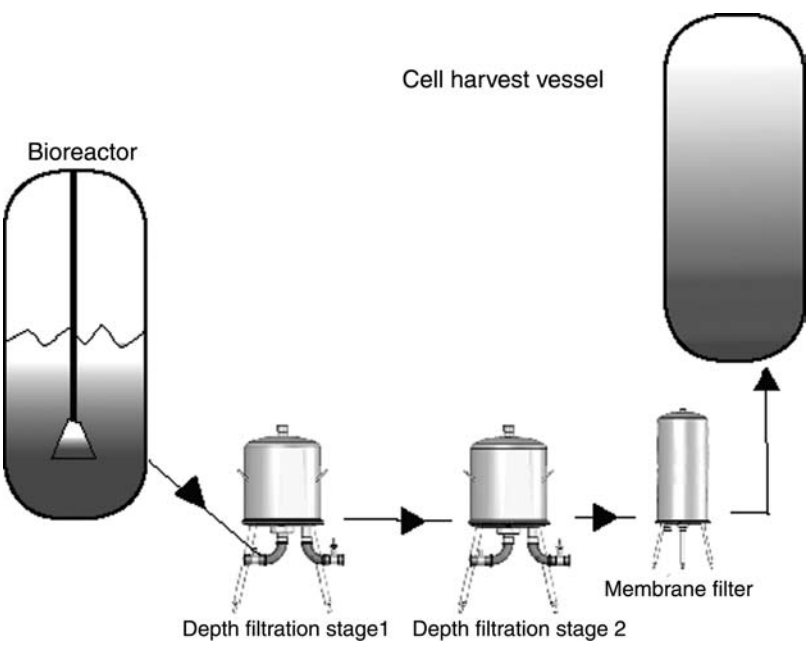


FIGURE 18 Fermentation filter system.

When running a forward flow test the filtrate can be tested for quality. Of course, if the filter sheet falls apart following the test or pieces of the filter are found in the soak fluid or the filtrate, the filter should be deemed to be incompatible with the fluid under the current process conditions.

By its definition a prefilter should remove sufficient particulate from the feed stream to protect the downstream filters for the duration of the entire batch. This can be determined by performing a particle analysis of the unfiltered fluid stream and then running trials with a filter sheet that fits the profile of the particle analysis. That filtered liquid should then be processed through the appropriate filter in the next stage of processing to assure the device will be adequately protected for the duration of the batch. If the particle size distribution is wide enough it may be necessary to include two stages of prefiltration, or a pre-filter to the pre-filter, in order to effectively prepare the fluid for the next process stage.

Filtration Trials and System Sizing

Choosing the proper depth filter and determining the required filtration area can be accomplished using basic laboratory filtration trials. First decide on the appropriate level of clarity, and then choose a grade of filter medium that should provide this clarity, as based upon either particle distribution data or manufacturer's recommendation. A number of filter grades might be evaluated before a selection is made. A minimum amount of the preparation being readied for processing is dedicated to trial testing. Properly performed, such evaluating tests can provide the data necessary to ensure the correct size filtration system for full-scale production.

Determine how much effective filtration area is required to perform the testing. The smaller the EFA, the less product need be expended to complete the testing. If the area of filter medium is too small, surface tension effects may results. A 47-mm diameter filter disk is a common trial-size for flat filter media. In a 47 mm diameter housing (i.e., 13.5 cm^2 of filtration area) every ml of product filtered can equate with 0.8 liters/m^2 of throughput in the production mode using appropriately larger filters.

The selection by trial can next be made of the filter medium in terms of porosity. The choice of a membrane filter of the correct pore size rating can be determined by testing several different possibilities. If necessary, compatibility can be assayed using membranes of different polymeric composition.

Cellulose-based depth filters can be formulated with a variety of filter materials, allowing for multiphase separations. As mentioned, if the primary goal of the filtration is color or catalyst removal, use an activated carbon-based filter medium, or an activated carbon treatment step in union with the filtration. For haze removal, DE-containing filter pads may provide the best results. Finally, for basic, general particulate removal, almost any formulation of filter pad can be used. Pre-test by first utilizing small volumes, approximately 50ml, of unfiltered liquid through the filter to determine whether the grade of medium is capable of providing the clarity,(often measured in NTU, Nephelometric Turbidity Units), that the process specifications require. The coarsest or highest flowing filter medium that fits this criterion, should be chosen for its suitability for scale-up testing.

In the struggle between productivity and the optimal ability of the filter to remove particulate, productivity usually wins. The lower the differential pressure during filtration, the longer the filter life will be in terms of total volume filtered per unit area; also, the more efficient the filter medium will be. However, most production processes operate under time and cost constraints. Thus, the operating pressures in the

scaled-up process will be greater than the ideal lowest possible pressure. For realistic results, many filtration tests are conducted at approximately 10psi (0.8 bar) to provide some balance between the two. Pressure can be supplied by a constant supply of compressed air or by a pump operating at a constant flow that can be set to supply 10psi of pressure. This method is acceptable with any low viscosity liquid. However, maintaining acceptable flow rates with liquids of viscosities higher than 100 centipoises, requires a more practical approach. Either an increase in pressure during the testing, or choosing a filtration device with a larger effective filtration area should be resorted to.

The bench scale trial testing should be considered finished when flow through the filter essentially stops or no longer conforms to a desired rate. During the testing, the length of time required to perform the filtration should be noted. When the testing is completed, two important informational items should be available, namely, the total throughput, that is, the amount of liquid that has passed through the filter. This will provide the volume per unit filtration area, using that specific filter media under the stipulated test conditions of: temperature, pressure, particulate content in the unfiltered liquid, and the fluid viscosity. The other important factor determined will be the flow capacity per unit area, or flux rate. These two calculations will allow the user to determine the filtration area and amount of time required to filter the entire production batch.

Batch sizing: $\mathrm{At} = 1/Tx\ V_b$ or flow rate sizing: $\mathrm{At} = 1/F_x \times F_s$ where: At = total required system filtration area; $T =$ throughput; $V_b =$ volume of batch; $F_x =$ flux rate and $F_s =$ system flow rate.

The two numbers rarely coincide; the more critical of the two most often determines the size of the filtration system required.

The same approach can be taken for the optimization of pre-existing filtration processes. If the current processes have not been addressed in many years, and are still operating under their original conditions, parameters may have changed. A new experimental investigation may disclose potential improvements. Perhaps EFA, the filtration area can be reduced; filtration costs saved, or faster batch times yielded. The trials can be performed at the end-user's site, at the supplier's local technical service facility, or at the filter medium manufacturer's laboratory. Attempts at updating processing operations merit encouragement.

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2
Charge Modified Filter Media

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INTRODUCTION

This chapter focuses on filtration developments, specifically charged filter media, as applied to primarily pharmaceutical and beverage applications. There are many examples where the use of charged filter media has been applied to additional fluid purification areas including microelectronics integrated circuit manufacture (Stone and Parekh, 1993), wastewater remediation (Hagg, 1998), water treatment (Mika et al., 1999), and others. Several reviews addressing the charged filtration media development and implementation are available in the literature (Andelman, 1995; Zeman and Zydney, 1996;). Further, the use of charge enhanced media has been applied to nearly the full range of filtration types, encompassing microfiltration, ultrafiltration, nanofiltration, and even reverse osmosis membranes (Ebersold and Zydney, 2004). Charged media has also been used in "non filtration" applications such as chromatographic separation (Zhou and Tressel, 2006).

Early developments to improve upon filter media comprising naturally occurring materials involved investigations to create synthetic filter media or "engineered" filter media consisting of natural components. The need to develop synthetic filter media was driven by requirements generated by the beverage and pharmaceutical industries. Prior to 1960s, pharmaceutical and beverage products were clarified or sterilized using filter media composed of cellulose and asbestos. Asbestos containing filters were used extensively in the sterile filtration of many pharmaceutical products, particularly biological products (e.g., fractionated blood products). Even after the advent of sterilizing membrane filters in the early 1950s, cellulose-asbestos prefilters were found necessary to reduce the contaminant loading on the membranes. In the production of fractionated blood products, the use of asbestos filters was uniquely critical for the removal of lipids, denatured protein, and bacterial endotoxins (pyrogens). The use of these filters dates back to a paper by Cohn et al. (1946) on the fractionation of human blood, the basis for what is now a major health industry. In the alcoholic beverage industry, cellulose-asbestos sheet media, or asbestos-based filter precoat mixtures, were used for final polishing and sterilization (Neradt, 1971; Rose, 1977). In the late 1960s, asbestos was being recognized as a potential health hazard and the drive to develop non asbestos containing filter media was born. Although the link to pulmonary disease was associated with inhalation of aerosolized fibers of asbestos (Selikoff and Lee, 1968), elimination of this material from filter media used for liquid filtration applications was being pursued. In 1973 the FDA

proposed restrictions on the use of asbestos filters in the manufacture of parenteral drugs (FDA, 1973) and issued appropriate regulations in 1975 (FDA, 1975 and 1976).

CHARGE MODIFIED DEPTH FILTER DEVELOPMENT

Most early (pre-1950) filter media consisted of natural materials such as cellulose and other fibers. These filter media were manufactured by mechanical means such as vacuum formed wet laydown methods to produce relatively thick filter sheets. This type of filter media is known as "depth filtration media" to distinguish it from more recent filter developments of membrane or surface filters involving precipitation of organic polymers.

It had long been recognized that adsorptive effects can enhance the capture of particulate contaminants. Contaminant particles and a porous filter medium can interact with two types of adsorptive forces: van der Waals forces, which are short-range and always attractive; and, the electrical double layer interactions, which may be attractive or repulsive depending on the surface charge of the contaminant particle and that of the pore surface. These interactions determine whether a particle will be attracted to or repulsed by the pore surface. If the contaminant particle and the pore surface are of the same charge, and the double layers of the particle and the surface interact in a repulsive manner, then the capability of a filter medium to remove particles physically smaller than the pores of the medium is limited. On the other hand, if the contaminant particle and the pore surface are oppositely charged, and there is at least no repulsive interaction between the electrical double layers of the particle and the pore surface, then particles physically smaller than the pore size of the medium are capable of being removed by adsorption by the process called electrokinetic capture. Since most contaminants encountered in nature are electronegative, this suggests that in order to increase retention of smaller particles, the filter medium should have a positive zeta potential.

As in 1971, an evaluation of asbestos had given strong indication that the unique filtration properties of asbestos depended on its electropositive surface charge. It became obvious that if a substitute were to be developed, it would have to be possible to modify the surface charge characteristics of other types of fibers and/or particulate materials. A review of the technical and patent literature revealed that the concept of chemically modifying the surface of a filter medium to provide enhanced filtration characteristics was not a particularly new one. The earliest reference was a 1936 patent (Cummings, 1936) that described the treatment of diatomaceous earth to produce an electropositive, insoluble aluminum hydroxide coating. Such treated diatomaceous earth, when used as a filter aid, acted as a flocculant for colloidal contaminants. In 1938, another patent (Elliott and Elliott, 1938) was issued describing the treatment of filter sand with rosin. While there is nothing in the chemistry of rosin that would lead one to believe that it could function as a charge modifier, the inventors appeared to believe that there was such an effect, and their patent states, "Sometimes this coating carries an electrical charge; either positive or negative, and opposite to that of suspended colloid particles." In 1952, a patent (Rodman, 1952) was issued describing the use of a modified aliphatic amine to treat filter media intended for use in oil filtration. That patent attributed enhanced sludge removal to cellulose fiber filters so treated.

In the early 1960s, synthetic polyelectrolytes were developed for use in water treatment. These polyelectrolytes are water-soluble, high molecular weight synthetic organic polymers containing a series of repeating monomeric units. The monomeric units are characterized by having ionizable functional groups that dissociate in water to

produce charged sites along the polymer chain. Initially, these polyelectrolytes were used as coagulants to improve the filterability of suspended solids. In such applications, they function by destabilizing colloid particles, through charge neutralization and/or bridging, so as to induce flocculation. In 1966 and 1967, Dow Chemical was awarded patents (Guebert and Laman, 1966; Guebert and Jones, 1967), which describe the use of cationic poly electrolytes to charge-modify filter aids and the use of such charge-modified filter aids in removing microorganisms from water. In the early 1970s, Bauman and his coworkers at the University of Iowa did extensive work on the use of polyelectrolytes as a pretreatment to provide an adsorbed charge modifier coating on filter aids and filter media (Baumann et al.,1970; Burns et al., 1970; Gulman et al., 1971). Their work is particularly significant because they were the first experimenters to use electrokinetic (streaming potential) measurements to quantify the level of charge modification achieved (Gulman and Baumann, 1964).

This prior technology demonstrated that it was feasible to charge modify the surfaces of normally anionic filter materials so as to produce a cationic surface characteristic and that such charge modification resulted in enhanced filtration properties. This knowledge allowed the definition of a set of developmental guidelines for the production of commercially useful depth filter media:

1. The basic filter material should be a fine, high surface area fiber or particulate with an ionic surface charge.
2. This material should be dispersible in a water slurry with cellulose fiber and being vacuum-formed into a thick sheet structure.
3. The chemical charge modifier should have a strong cationic functionality and should be capable of being chemically bonded to the anionic high surface area substrate.

This latter requirement was based on the needs of the pharmaceutical and beverage industries for a “clean” (low extraction) and “safe” (no toxicological problems) filter medium. Finding the appropriate charge modifier system to satisfy this requirement was the innovative step that moved development beyond all of the prior technology, which was based on the adsorption of a charge-modifying agent.

Unfortunately, adsorption is a reversible process, and such adsorbed charge modifiers exhibited high extraction levels. In practice, this meant that the charge modifying agent must have an appropriate cross-linking functionality in addition to its cationic functionality. During the period 1972–1976, several basic types of filter media employing this method of creating charge enhanced filter media were developed and commercialized (Ostreicher, 1977a, b, 1982; Hou and Ostreicher, 1991; Ostreicher and Hou, 1981).

Success in duplicating the performance of cellulose-asbestos filter media is most easily demonstrated by comparing the laboratory performance of a Zeta Plus media grade (Fig. 1A) with that of the corresponding Seitz cellulose-asbestos grade (Fig. 2B). These laboratory results were soon verified by the success of Zeta Plus in replacing cellulose-asbestos in a broad range of industrial filtration applications.

Because of the low wet strength of cellulose-asbestos, this medium had been limited to use as a sheet medium in filter presses, with all of the associated problems of leakage, sterilization, and lengthy change out time and correspondingly high labor costs. In addition to having equivalent or superior filtration performance, Zeta Plus exhibited significantly better wet strength. This was an additional benefit of the cross-linking characteristics of the charge modifier systems used, and it allowed the development of a new generation of radically improved disposable depth filter cartridges. In the intervening years, such cartridges have significantly displaced the use of filter presses.

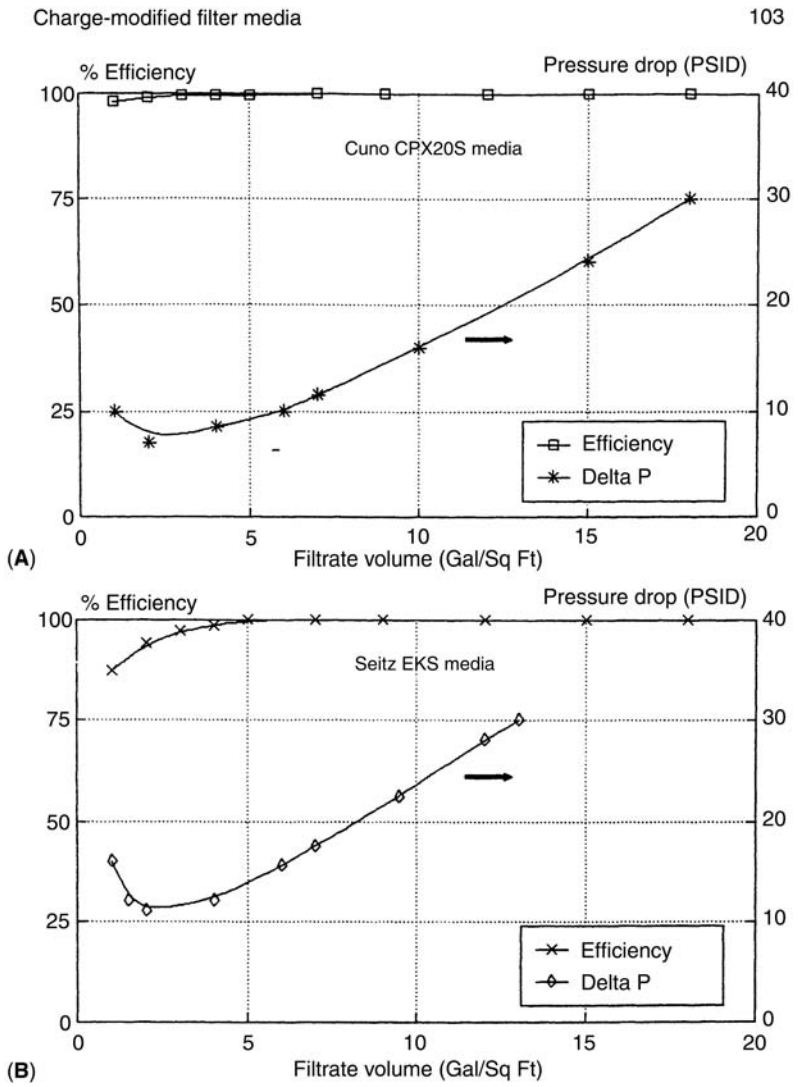


FIGURE 1 (**A**) Comparative filtration characteristics for Zeta Plus medium. (**B**) Comparative filtration characteristics of cellulose-asbestos medium.

POLYMERIC MICROFILTRATION MEMBRANE DEVELOPMENT

Rising out of the development of positively charged depth filter media, the concept of charge modification of polymeric microporous membranes was first proposed in the early 1970s by Ostreicher. It was believed at that time that there was no advantage to trying to embellish the performance of an "absolute" filter that was already capable of achieving the level of performance demanded by the application. In the late 1970s, however, the reliability of such membrane filters came into question with several investigators reporting bacterial passage through such supposedly "absolute" membranes (Wallhausser, 1979; Howard and Duberstein, 1980). In addition, the medical industry was coming to realize that other contaminants besides bacteria, viruses and bacterial endotoxins (pyrogens), had to be removed from parenteral drugs for patient

safety (Brown, 1980). Significant success was achieved in removing both viruses and bacterial endotoxins (Sobsey and Jones, 1979; Gerba et al., 1980; Hou et al., 1980) by use of the charge-modified Zeta Plus filter medium, and it appeared that such enhanced performance could also be achieved with a charge-modified polymeric membrane. The new nylon membrane charge-modified filter media were based on the unique morphology and surface chemistry of nylon (Ostreicher et al., 1984) and the very specialized requirements of the pharmaceutical industry (low organic extractions) and the semiconductor industry (low-ionic extractions). This necessitated the development of two new two-component charge modifier systems (Barnes et al., 1984; Ostreicher et al., 1984).

The primary market, however, for such membranes was in filtration and sterilization of pharmaceutical products. In this market, microporous polymeric membranes of 0.2 μm nominal rating have found widespread use in the sterilization of parenteral drugs. For this purpose, their performance was characterized in terms of the complete removal of the microorganism *Pseudomonas diminuta* (Pall, 1975).

The existence of the adsorptive phenomena in conventional microporous membranes is recognized (Tanny and Meltzer, 1976; Tanny et al., 1979). The effect of such adsorptive phenomena on the performance of "sterilizing" membranes was treated as a secondary and unremarkable attribute of the base polymer used to produce the membrane. Perhaps exaggeratedly, mechanical sieving was defined as the primary mode of filtration. All of the commonly accepted modes of membrane characterization concerned themselves with the determination of pore size distributions (especially maximum pore size) and the correlation of such physical parameters with the bacterial rejection characteristics exhibited by the membranes.

Historically, membranes were characterized as surface-type filters. In an SEM study, Knight et al. (1982); were able to demonstrate rigorously that a charge-modified nylon membrane functions as an adsorptive depth filter by virtue of the electrokinetic capture and adsorption function provided by the combination of charge modification and high internal membrane pore surface area. Figure 2 shows a cross section of such a charge-modified membrane after it was subjected to a 0.109 μm monodisperse latex (MDL) bead challenge. This photograph demonstrates the reality of electrokinetic capture and adsorption with the MDL particles adhering to the surfaces of the relatively larger pore structure. That such removal is quantitative is shown graphically in Figure 3, which presents the particle removal efficiencies of a charge-modified 0.2 μm membrane and unmodified 0.1 and 0.2 μm membranes. The pressure drop characteristics for each of these membranes are shown superimposed on the efficiency curves in Figure 4.

Initially, Cuno was the only filter manufacturer producing a charge-modified filter medium. Pall Corporation (nylon 66), Gelman, and Millipore have since joined in this technology. Gelman produced a positively charged polyethersulfone sulfate membrane, while Millipore produces a charge-modified (poly vinylidene difluoride; PVDF) membrane.

THEORETICAL BASIS OF CHARGE MODIFIED FILTER MEDIA

New test methods had to be developed to evaluate and quantify the performance of candidate charge-modified media and, eventually, to provide process and quality control criteria for the final product. The primary performance characteristics for any filter

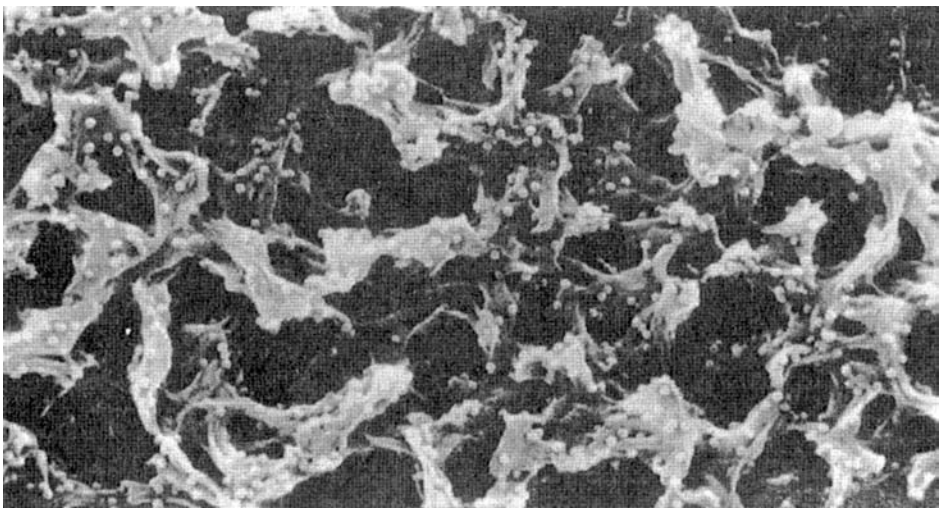


FIGURE 2 Electrokinetic capture of 0.109 μm particles on a charge modified membrane.

medium are capture efficiency and capacity. The test techniques that had been developed to measure these characteristics in unmodified submicrometer filter media, such as bubble point, diffusion flow, and sterilization testing with specific microorganisms, dealt strictly with mechanical straining. They were not useful in measuring the efficiency and capacity of charge modified media.

It was necessary that the contaminant particle size be smaller than the pore size of the medium being tested. In addition, it was necessary that the contaminants used have consistent and reproducible surface charge and particle size distribution characteristics. These requirements were necessary so that consistent and reproducible evaluation could be made of the electrokinetic capture and adsorption characteristics of the media independent of mechanical straining effects. The following test methods were developed to meet these requirements.

Monodisperse latex bead testing, the "workhorse" of the developmental efforts, consists of challenging the filter medium sample, at a specified constant flow rate, with an aqueous dispersion of single-size monodisperse polystyrene latex beads adjusted to a specified turbidity, pH, and resistivity. The effluent turbidity and medium pressure drop are measured and recorded as a function of the throughput. The particle removal efficiency of the media sample, for the specific particle size used, can be calculated directly from the inlet and effluent turbidity values (Knight and Ostreicher, 1981). These tests use relatively high contaminant challenge levels to reduce the time to breakthrough to an acceptable value for reasons of practicality as shown in Figure 5. It is evident from the curves shown in this figure that at modest challenge concentrations, one is dealing with a billion particles per milliliter to evaluate filter media. It is also evident that to evaluate media with different sizes of MDL beads, a constant number of particles should be used rather than a constant concentration. These MDL particles are commercially available in a number of diameters ranging from 0.021 to $> 2.0\,\mu m$. The results shown in Figures 1, 3, 6, 7, and 8 are based on the use of this particular MDL bead test technique.

As developmental efforts led to the surface charge modification of ever finer pore size media, even the MDL test was impacted by interference from mechanical straining effects. To deal with this situation, a test technique was developed based on the use of

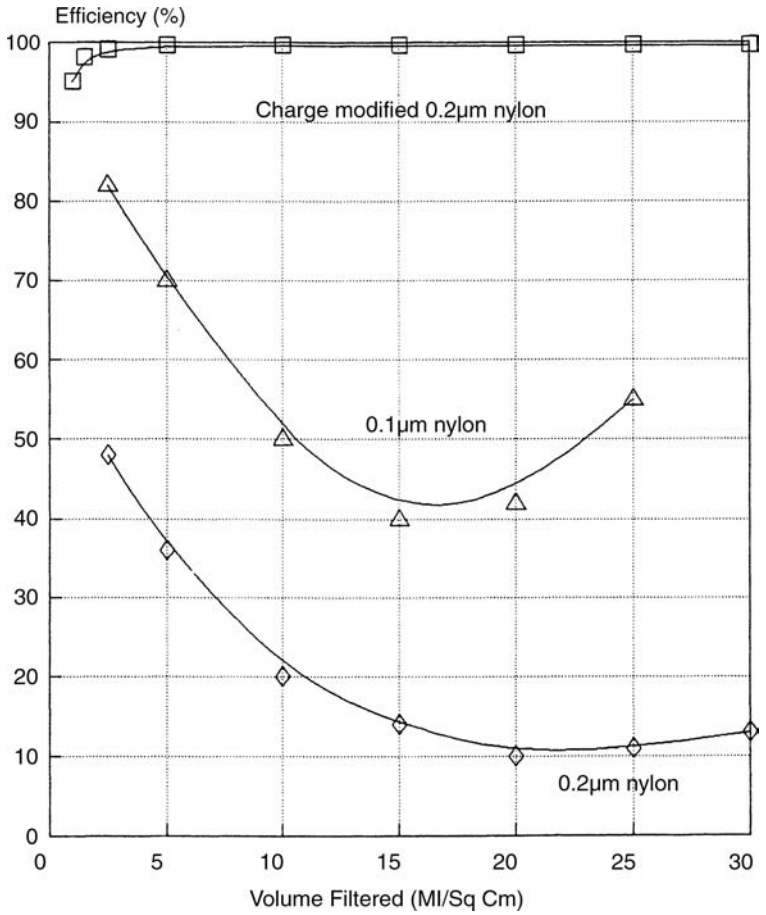


FIGURE 3 Filtration characteristics of modified and unmodified nylon 66 membranes, subjected to 0.109 µm MDL bead challenge.

metanil yellow, a water-soluble anionic dye with a molecular weight of approximately 375. The dimensions of this molecule are approximately 18 Å× 9 Å (Graham, 1955), allowing it to be used as a test contaminant for almost any charge-modified media down into the microfiltration range without interference from mechanical straining effects. In this test method, the media sample is challenged, at a specified constant flow rate, with an aqueous dispersion of metanil yellow adjusted to a specified pH and resistivity, and light transmittance is measured with a spectrophotometer. The light transmittance of the effluent is measured and recorded as a function of throughput.

Surface charge cannot be measured directly or quantified by indirect techniques. Such indirect techniques as streaming potential (Knight and Ostreicher, 1981) for porous filter media allow the measurement of the potential at the hydrodynamic shear plane (zeta potential). The zeta potential is an important indicator of the electrokinetic status of a filter material and can be used to determine the capacity of a medium for contaminant and to determine its efficiency, as is shown later. Zeta potential is defined as the potential at the surface of shear between a liquid and a particle or a surface. It cannot be directly measured but can be calculated, for example, from streaming potential measurements, which is one of a series of four electrokinetic effects, the others being electrophoresis,

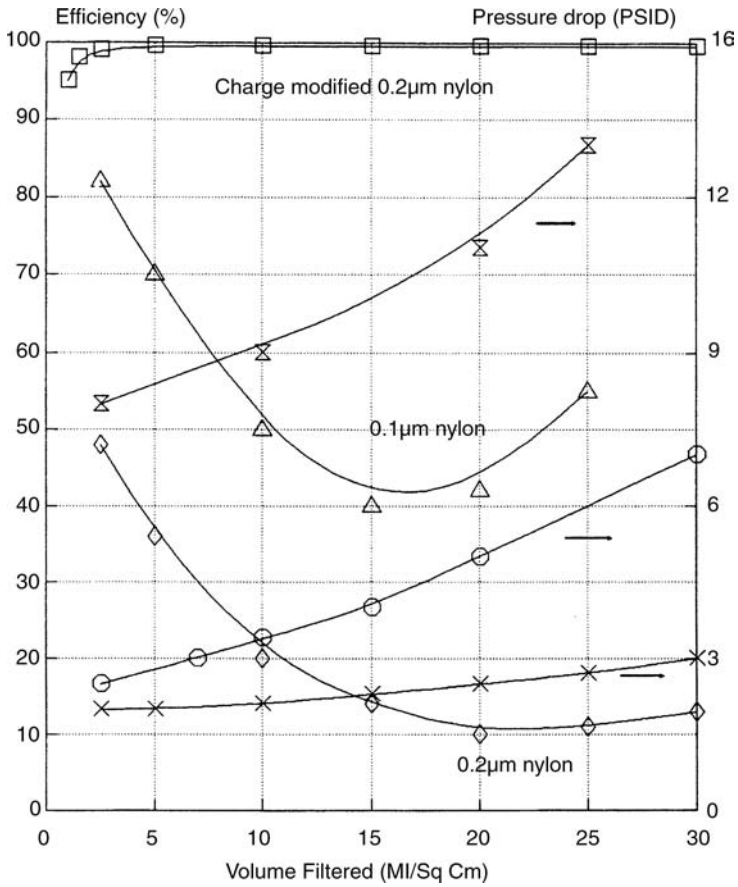


FIGURE 4 Filtration characteristics of modified and unmodified nylon 66 membranes subjected to 0.109 μm MDL bead challenge. ⧖, ΔP 0.1 μm nylon; ○, ΔP charge modified 0.2 μm nylon; ×, 0.2 μm nylon.

electroosmosis, and sedimentation potential. All these effects are due to the disturbance of the static equilibrium conditions in the electrical double layer that exists at a solid/liquid interface.

The capillary pore model is used to demonstrate the concept of charge-modified membrane and is shown in Figure 7. In this case, the capillary represents one pore of a network of pores. The wall of the capillary is shown as having a layer of positively charged ions attached to the capillary wall, with an equal number of ions of opposite charge loosely distributed in the second layer. The surface of shear, as indicated in Figure 7, creates an imbalance in charge due to laminar flow of fluid through the capillary.

Streaming potential is the electric potential that is developed when a liquid is forced through a capillary or a network of capillaries such as a microporous membrane. It is due to the pressure drop across the end of a capillary or a porous plug such as a microporous membrane containing a liquid. The fluid flow created by the pressure drop across the capillary creates a disturbance in the electrical double layer, which sets up an electric potential across the ends of the capillary or porous medium. This potential can be measured directly by using a pair of inert electrodes in an external circuit of high

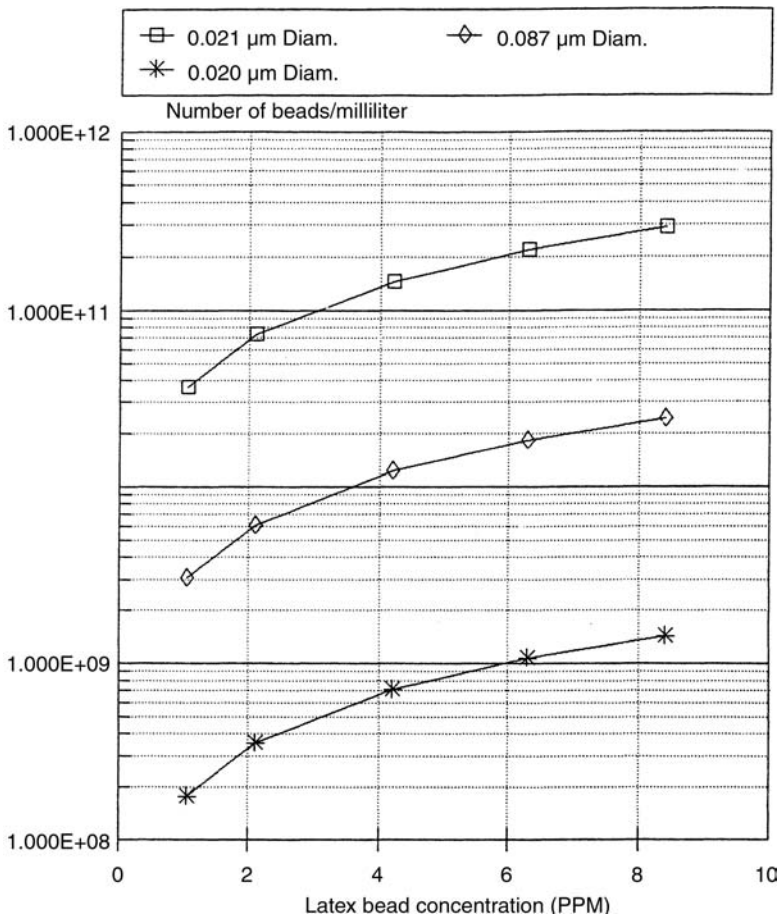


FIGURE 5 Number of latex per milliliter vs. latex bead concentration for beads of different diameters.

impedance, so that all the current is forced to flow back through the system being measured, in this case a capillary. An apparatus for measuring streaming potential on a charged glass capillary is shown in Figure 8. It should be obvious from Figure 8 that a simple test apparatus can easily be assembled to evaluate the charge characteristics of filter material, whether it be microporous membrane, felted filter medium, or granular material.

The relationship between the zeta potential and the surface potential is, however, a subjective one that depends on the ionic species and their concentrations in the test fluid. These test techniques are suitable for qualitative evaluations of the filtration mechanism and the effect of pH on both the charge modification process and the filtration process.

The MDL bead and dye tests are capable of providing a quantitative measure of the adsorptive capacity of a charge-modified filter medium. For reasons that are discussed later, the test results are sensitive to both the ionic content (species and concentration) and the pH of the test contaminant dispersion. Precise control of these two factors is critical to the reproducibility of the test results. Because of the abbreviated time frame, they are sensitive to the initial short-term pH shift and ionic extractions induced by the filter medium sample. Streaming potential measurements (Knight and Ostreicher, 1981) show

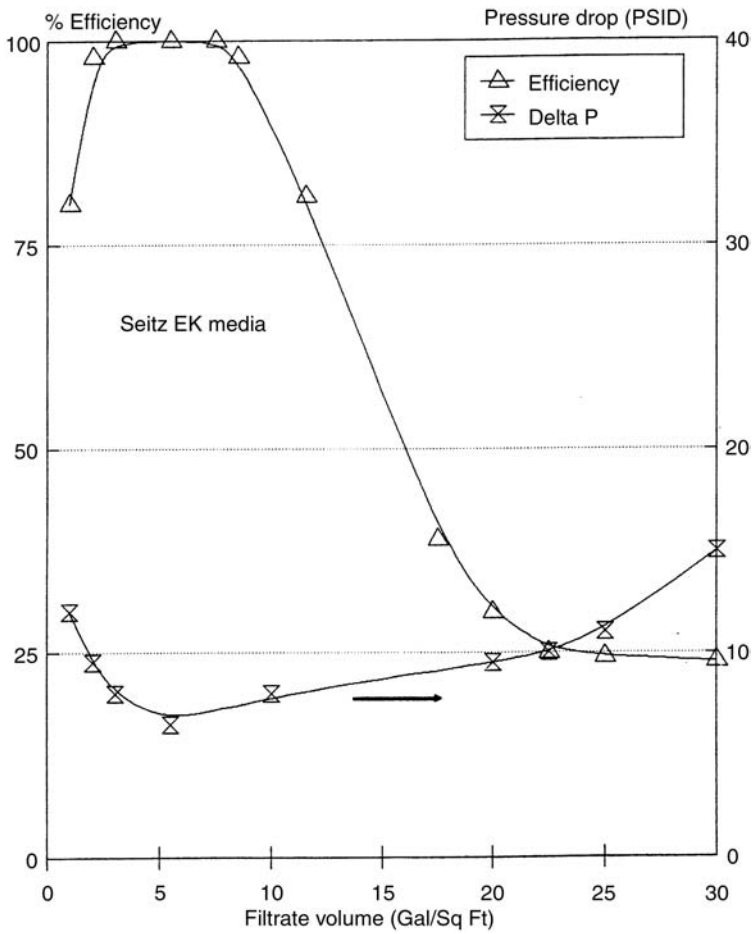


FIGURE 6 Filtration characteristics of cellulose-asbestos filter medium.

that a potentially significant period of time is required for the media double layer to come into equilibrium with the bulk solution. Experimental precision requires that these factors be recognized and dealt with accordingly.

The electrokinetic behavior of colloidal particles, especially as it influences particle interactions and stability, has been the subject of a great deal of theoretical and experimental investigation, and there is an extensive literature available dealing with this aspect of electrokinetic phenomena. Unfortunately, no similar effort has been expended to investigate the electrokinetic interactions between colloidal particles and porous filter media. Cuno investigators were able to develop a "working" model of these interactions based on empirically derived understandings.

Wnek (1979), using the electrokinetic perspectives of colloidal chemistry, proposed that asbestos owed its unique filtration characteristics to the strong positive surface charge that it possessed at neutral and lower pH. He further proposed an operative filtration mechanism for such positively charged filter media that consisted of electrokinetic capture (via attractive double layer interaction) and adsorption of negatively charged particles, which in turn resulted in modification of the surface charge on the media from positive to negative. This, he further proposed, would

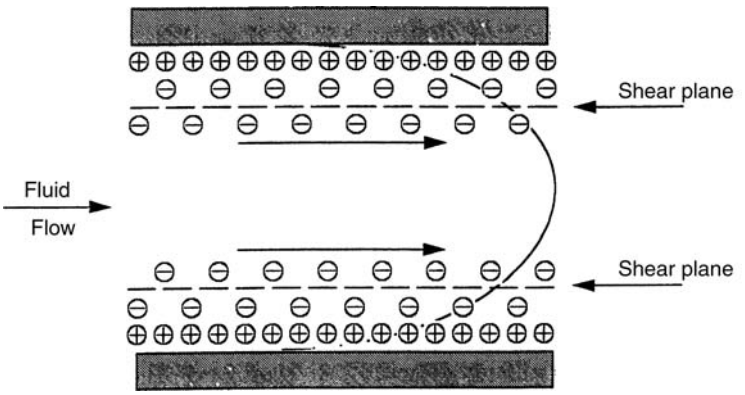


FIGURE 7 Ion distribution near the wall of a positively charged capillary.

eventually inhibit additional electrokinetic capture, allowing eventual breakthrough of the contaminant particles.

With the development of the previously described MDL bead test, it became possible to experimentally verify the breakthrough phenomena for cellulose-asbestos filter media (see Fig. 6) and, subsequently, for other forms of charge modified filter media as shown, for example, in Figures 1, 3, and 7.

Further testing combined the MDL bead test with streaming potential measurements (Knight and Ostreicher, 1981). This use of simultaneous streaming potential and particle removal efficiency measurements allowed the defining of the real-time relationship between the electrokinetic condition of the media and the particle removal efficiency, as shown in Figure 9. For a positive surface charge modified depth filter (Zeta Plus CPX-50S), the 0.109 μm negatively charged mono. The capillary pore model is used to demonstrate the concept of charge-modified membrane and is shown in Figure 7. In this case, the capillary represents one pore of a network of pores. The wall of the capillary is shown as having a layer of positively charged ions attached to

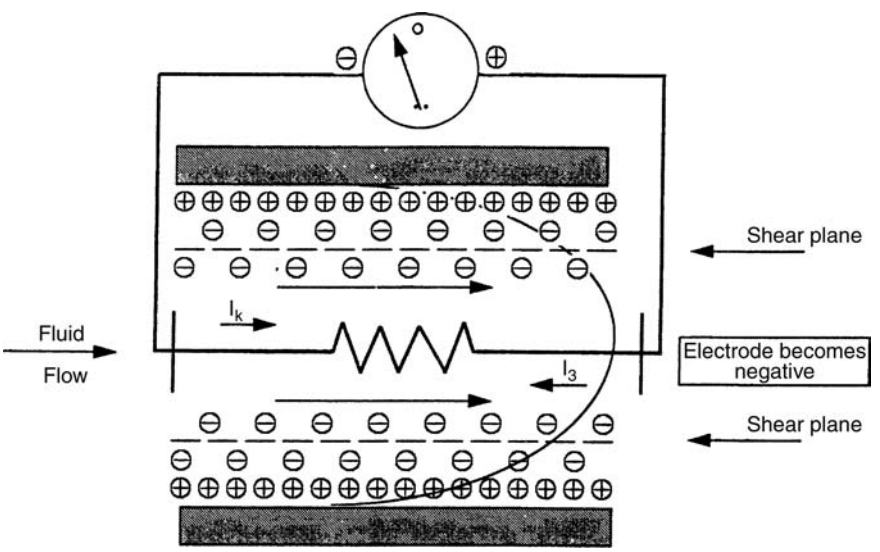


FIGURE 8 Ion distribution near the wall of a positively charged capillary with measuring circuit.

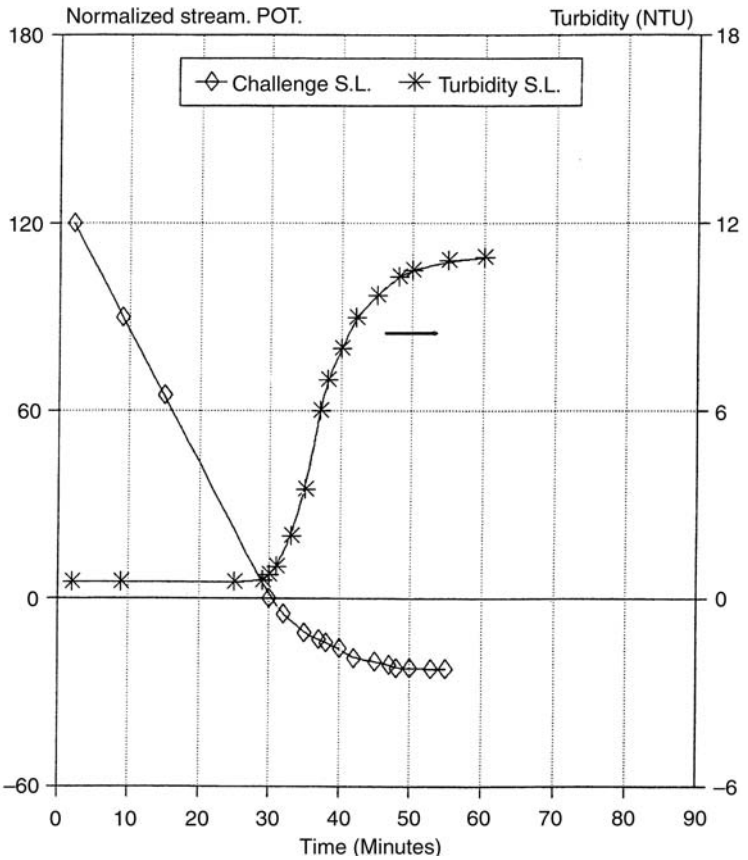


FIGURE 9 Results of 0.109 μm MDL bead challenge to single-layer type 50S Zeta Plus medium.

the capillary wall, with an equal number of ions of opposite charge loosely distributed in the second layer. The surface of shear, as indicated in Figure 11, creates an imbalance in charge due to laminar flow of fluid through the capillary. kinetic capture and adsorption. The interaction between the constant-rate latex bead challenge and the medium gives a normalized streaming potential that decays linearly from a negative value (positive zeta potential) through zero and then asymptotically approaches a final positive normalized streaming potential (negative zeta potential). Only when the normalized streaming potential approaches and passes through zero does the particle removal efficiency, as measured by effluent turbidity, start to decrease. Finally, the particle removal efficiency asymptotically approaches zero (inlet turbidity = effluent turbidity) as the normalized streaming potential similarly approaches its final value. These results appear to verify the model proposed by Wnek, perhaps subject to some minor modifications. On the basis of Wnek's model, it was anticipated that the streaming potential would asymptotically approach zero with contaminant breakthrough starting at a low negative normalized streaming potential and going to zero filtration efficiency at zero streaming. These tests, however, showed that the filtration efficiency did not approach zero until the normalized steaming potential approached the high positive values exhibited by the unmodified filter medium. This actual performance appears to be indicative of a discrete, rather than uniform, positive charge deposition resulting in a grossly amphoteric surface, that is, a surface possessing a heterogeneous distribution of anionic and cationic charge functionalities.

At first glance, it would appear that the adsorptive capacity of a medium might be predictable from the initial equilibrium value of the normalized streaming potential. To examine this question, a test series was run comparing the streaming potential and 0.109 μm MDL capacity of single- versus double-layer media samples. The results, shown in Figure 10, demonstrated that although the adsorptive canacitv is additive, the normalized streaming potentials for the single-layer and double-layer media samples were identical. At least in this case, therefore, the normalized steaming potential did not correlate with the adsorptive capacity. The correlation between effective medium thickness and adsorption capacity did appear to indicate that total charge-modified surface area might be the primary determinant of capacity.

Early characterizations of the surface charge characteristics of asbestos (see Fig. 11) show a strong relationship between the electrokinetic characteristics and the pH of the water in which the asbestos fibers were dispersed. This relationship is the result of the effect of pH on the relative levels of dissociation of the various surface functional groups that, in a composite, determine the surface charge characteristics. More often than not, the surface chemistry of the systems with which we are dealing are amphoteric; that is, both anionic and cationic functional groups exist at the surface.

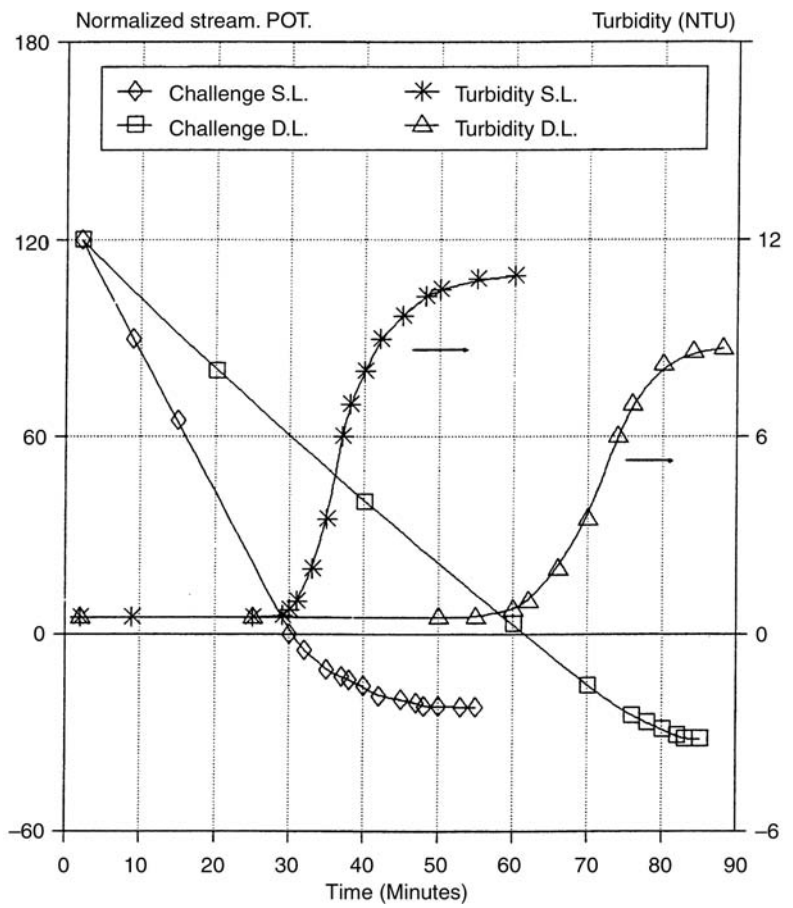


FIGURE 10 Results of 0.109 μm MDL bead challenge to single- and double-layer type 50S Zeta Plus medium. *Abbreviations*: S.L., single layer; D.L., double layer.

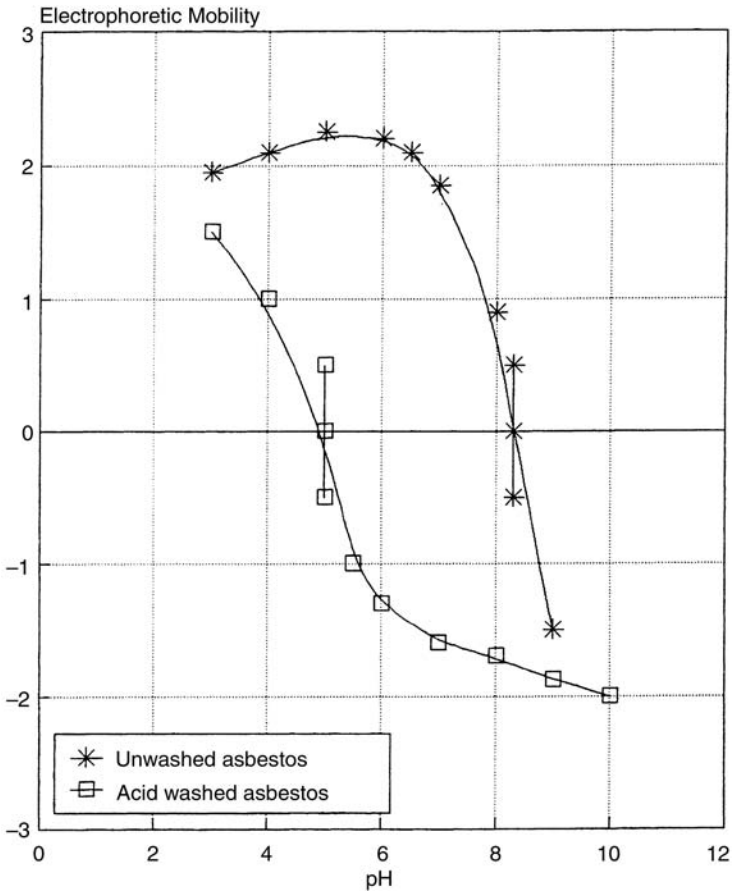


FIGURE 11 Electrophoretic mobility of chrysolite asbestos.

Increasing the pH will suppress the dissociation of the cationic groups and increase the dissociation of the anionic groups, and vice versa.

Unmodified nylon 6,6 membrane, for example, has surface charge characteristics determined by the carboxyl and amine end groups of the nylon 6,6 molecule as shown conceptually in Figure 12 and assumes that the carboxyl and amine end groups are uniformly distributed throughout the wetted pore surface.

In Figure 13, it is shown that the unmodified nylon 6,6 membrane exhibits a negative surface charge at pH > 6.0. Charge-modifying the nylon membrane surface with a strong quaternary amine charge modifier elicits a radical change in this characteristic, providing a positively charged surface which is stable up to pH 11.0.

One might expect that electrokinetic capture and adsorption performance would be impacted by pH, and in fact this is the case. The results of a metanil yellow dye challenge test on nylon microporous membrane are shown in Figure 14. The effect of pH and type of charge modifier on membrane metanil yellow dye capacity is clearly shown.

Understanding the impact of pH on the electrokinetic capture and adsorption characteristics of a surface charge modified filter medium involves recognition that the surface charge characteristics of the medium and the contaminant change with pH. Given this combined effect, it becomes evident that pH can be adjusted to enhance and optimize

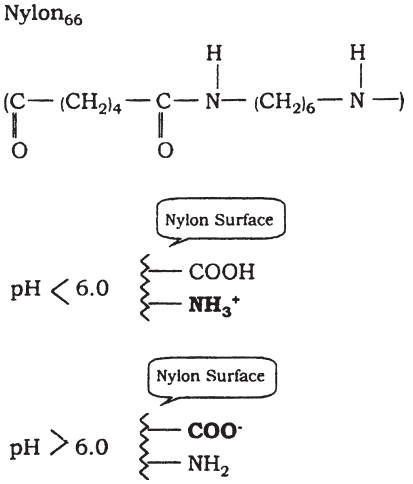


FIGURE 12 Surface charge characteristics of nylon 66.

the performance of charge-modified filter media, and, indeed, such techniques have been used in a number of applications.

The ionic environment plays an important role in the adsorptive capacity of charge-modified filter media. The size of the ionic double layer surrounding a charged particle or surface is controlled by the concentration and valence of the counterions in the solution. Riddick (1968), measured the electrophoretic mobility for a silica colloid in various concentrations of various electrolytes. The colloid concentration used was 100 ppm. Zeta potentials were calculated from the mobility values and were plotted against electrolyte concentration. The curves are shown in Figure 15. Inorganic electrolytes can have a significant impact on the zeta potential. In this example, the zeta potential of a dilute solution of colloidal silica was modified by adding different electrolytes. The trivalent cation of aluminum chloride easily sends the zeta potential from a negative value toward a positive value, while the potassium sulfate, a monovalent cation, goes more negative until a plateau is reached. The zeta potential begins to rise because the high-ionic concentration begins to compress the double layer.

Increased ionic concentration and higher valence counterions reduce the thickness of the double layer, and vice versa. This has a significant impact on the repulsive or attractive interactions between particles and between a particle and the filter pore surface. The net effect of ionic concentration and valence can be, for evaluative purposes, quantified in terms of the resistivity of the solution. Decreasing the resistivity of the solution will increase the adsorptive capacity of a charge-modified filter medium. It does so, apparently, by reducing the thickness of the double layer surrounding the contaminant particles. Since there is a repulsive double-layer interaction between particles, this reduction allows the particles to approach each other more closely and decreases the amount of "space" occupied by an adsorbed particle on the oppositely charged filter medium pore surface. In one particularly interesting test, a Zeta Plus sample was subjected to a low conductivity 0.109-μm MDL bead challenge and run until breakthrough occurred. At that point, a salt was added to the contaminant dispersion to significantly increase the conductivity. As shown in Figure 16, the effect of this salt addition was to restore the ability of the sample to absorb contaminant particles.

Charge-modified cartridges have demonstrated an exceptional ability to retain high concentrations of particles in semiconductor UPW systems. The electropositive

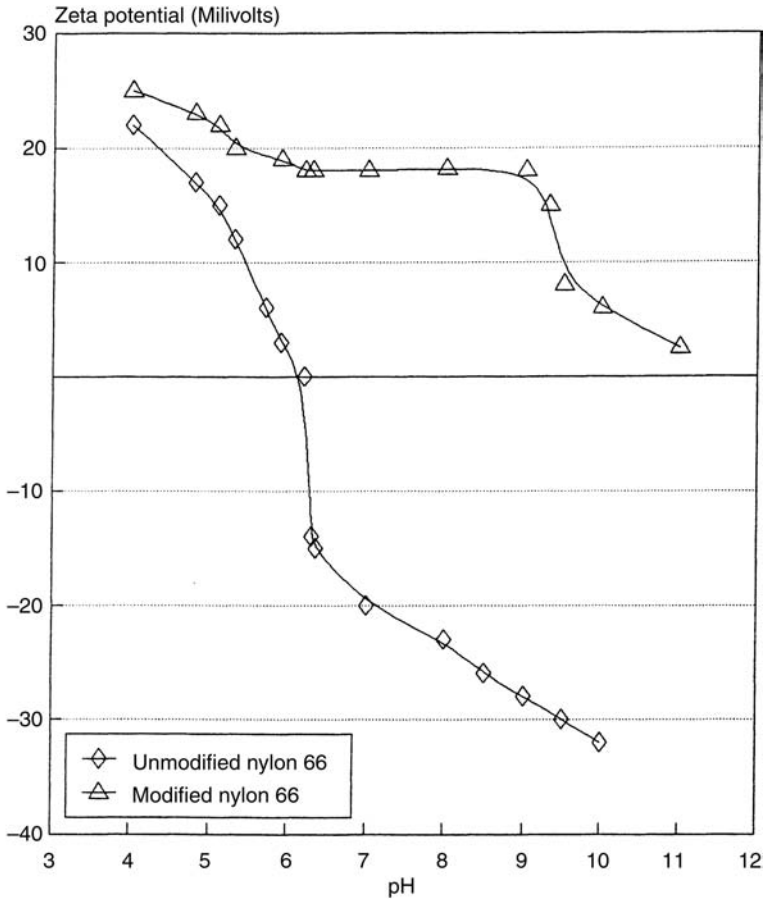


FIGURE 13 Electrokinetics of charge-modified and unmodified nylon 66—zeta potential vs. pH.

charge modification to the nylon microporous membrane enables such cartridges to remove particles much smaller than the rated pore size of the membrane. The electrokinetic adsorption and mechanical sieving have proven to be effective in removing contaminants such as colloidal silica and fragmented ion-exchange resin beads from UPW systems. The ability of such 0.04-μm membranes to adsorb 0.021-μm MDL beads is illustrated in Figures 17 and 18.

Figure 17 shows the equilibrium flush-out of a 0.04-μm Zetapor microporous membrane subsequently challenged with a 4.2 ppm (1×10^{11} beads/ml) suspension of 0.21 μm MDL beads. Figure 18 is a scanning electron photomicrograph of the membrane challenged in Figure 17.

RECENT APPLICATIONS OF CHARGED MEMBRANES

The development of pharmaceuticals produced by biotechnology has created an increased need for critical separations. Biologic therapeutic pharmaceuticals are complex, high

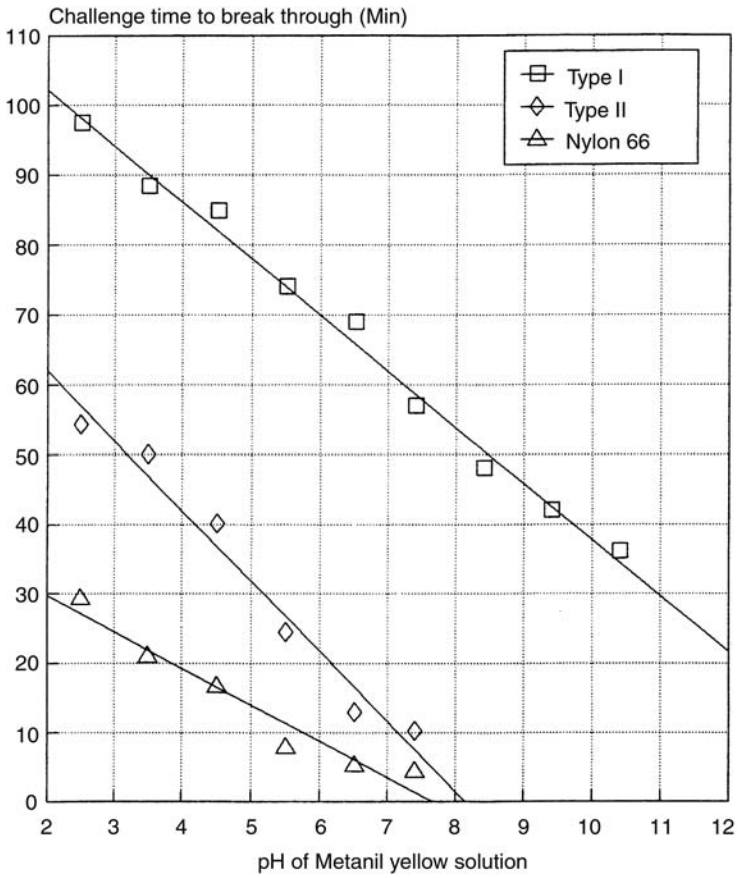


FIGURE 14 Effect of charge modification type on metanil yellow challenge capacity—pH vs. time to breakthrough.

molecular weight amphoteric compounds. Charge modified depth filters, microporous membranes and ultrafiltration membranes have been developed to facilitate separation and purification of these complex biomolecules. The paragraphs below reference some recent applications of charged microporous membranes and depth filters in biological separations. Referred to are endotoxin and DNA removal, clearance of virus from biological solutions, discrete separations of low molecular weight proteins and peptides by charged ultrafiltration membranes and removal of protein aggregates and contaminating host cell proteins (HCPs) from monoclonal antibody preparations. In addition to these applications, there are emerging separation applications involving membrane chromatography (Zhou and Tressel, 2006) and other methods of effecting charged based separations including pore filled membranes. Although these recent developments are beyond the scope of this chapter, several references are provided (Brandt et al.,1998; Charcosset, 1999).

Charge-modified filter media are now widely used in the pharmaceutical industry for the removal or control of negatively charged contaminants such as endotoxins. Carazzone et al.(1985) investigated charge-modified filter media to determine if they were able to retain substances of opposite charge in solutions of different composition,

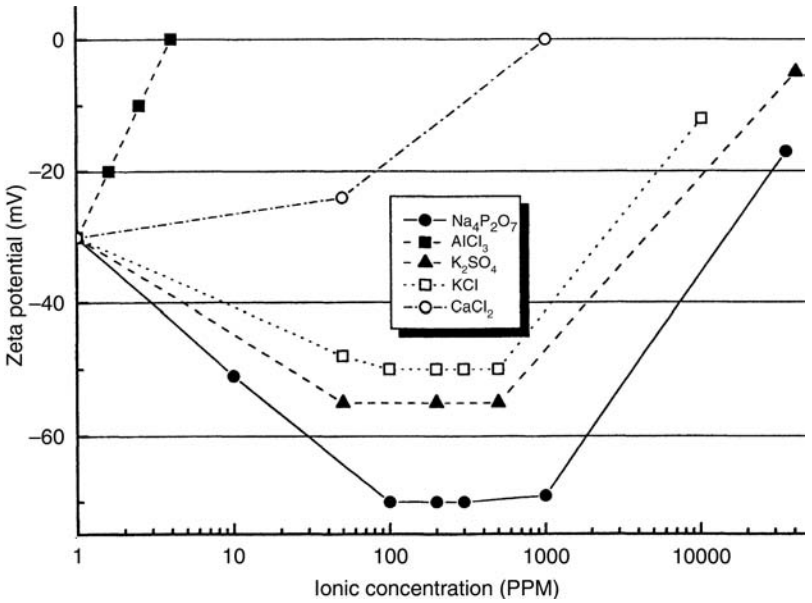


FIGURE 15 Zeta potential vs. concentration curves. *Source*: Reconstructed from Riddick, 1968.

that is, in both electrolytes and nonelectrolytes. The endotoxin used in this study was extracted from gram negative *Escherichia coli* B5. Carazzone et al. found that (1) the removal of pyrogens to below detectable limits from distilled water is possible using both 0.2 and 1.2 μm charge-modified membranes; and (2) in the presence of 0.9% NaCI solution, neither the 0.2 nor 1.2 μm membrane depyrogenated the challenge solution and, similarly, neither set of charge-modified membranes removed the endotoxin in a 2% peptone solution.

Evaluation of the same charge-modified filters with Serratia marcescens produced similar results in that organism retention was higher for charge-modified filters than for conventional membrane filters and the presence of electrolytes or peptone significantly reduced the efficiency of the charge-modified membrane.

Carazzone concluded that: "the efficiency of positively charged filter media to remove pyrogenic substances and to improve microorganism removal from solutions varies with the composition of the solution to be filtered and with the pre-filter and final filter used."

Wickert (1993) reviewed the work of Barker and coworkers, who conducted a validation study to quantify the endotoxin removal capacity of charge-modified filters in water systems while evaluating the effect of flow rate, temperature, and steam sterilization conditions. The test system consisted of a 0.45-μm positively charged prefilter, a 0.2-μm positively charged filter to remove the endotoxin, and an additional 0.2-μm positively charged filter to retain endotoxin that might break through. All three filters were connected in series. The system was challenged with *E. coli* B5 purified endotoxin suspended in 0.5 mM EDTA at a challenge level of 8×10^7 EU. Variations in pH >3 did not significantly affect the filter performance, but reducing the flow rate appeared to increase the filters' capacity for endotoxin. The effect of temperature and steam sterilization interval did not have an effect on endotoxin removal and therefore did not affect the stability of the charge modifier. Wickert concludes, "the information on the effect of varying process parameters also demonstrates the capability of this technology and confirms its flexibility."

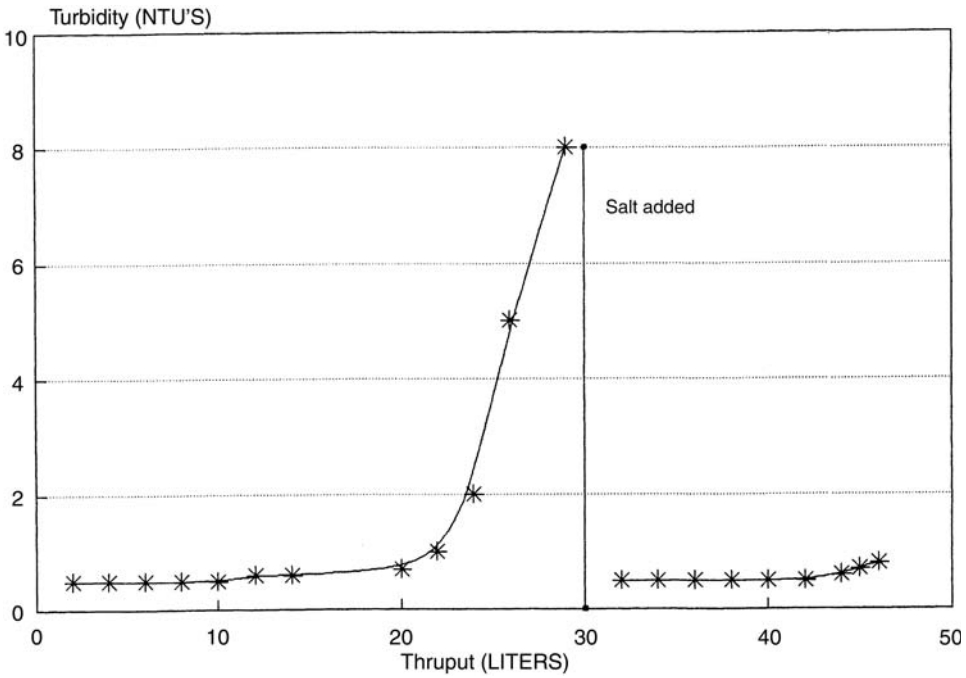


FIGURE 16 Effect of salt addition on adsorptive capacity.

Nagasaki (1988) evaluated nylon 6,6 charge-modified and unmodified filter cartridges after 5 months of identical service in a semiconductor high purity water system. He found that one of the key benefits of charge modification is that the membranes are much less susceptible to hydrolysis, speculating that the protection mechanism was the cross-linking of the charge modifier and the nylon. Hot water exposure testing was performed on both charge-modified and unmodified membranes. The result showed that the charge-modified membranes maintained elasticity, whereas the unmodified membranes became brittle after 2000 h of exposure. He concluded that "the added stability of the charge modified membrane is therefore another important factor to be taken into account in selecting a filtration system."

In a study of calf thymus DNA retention by depth filters published by Dorsey et al. (1997), the authors found depth filter mediated retention of DNA was attributed to both mechanical separation and electrokinetic attraction. The data showed, however, that the primary retention mechanism of nucleic acid was due to electrokinetic forces. Figure 19 shows retention of 100 ng/ml calf thymus DNA at pH 7.4 using depth filter media with varying concentrations of cationic resin. Percent retention of DNA increases with increasing resin content to a maximum of 5% resin. Further DNA retention was believed to be limited by steric hindrance. Retention of nucleic acids is significant to biotherapeutic protein purification as production methods involve the use of cell culture and contaminating host cell nucleic acids require removal to low levels in fished drug product.

In addition to use in cell clarification and bioburden reduction, depth media has been shown to be useful during bioprocessing application to remove HCPs and nucleic acids. A study performed by Yigzaw et al. (2006) demonstrated that depth media having a high level of positive zeta potential was able to remove HCP to a level that protected chromatography columns downstream and reduced the frequency of protein aggregation.

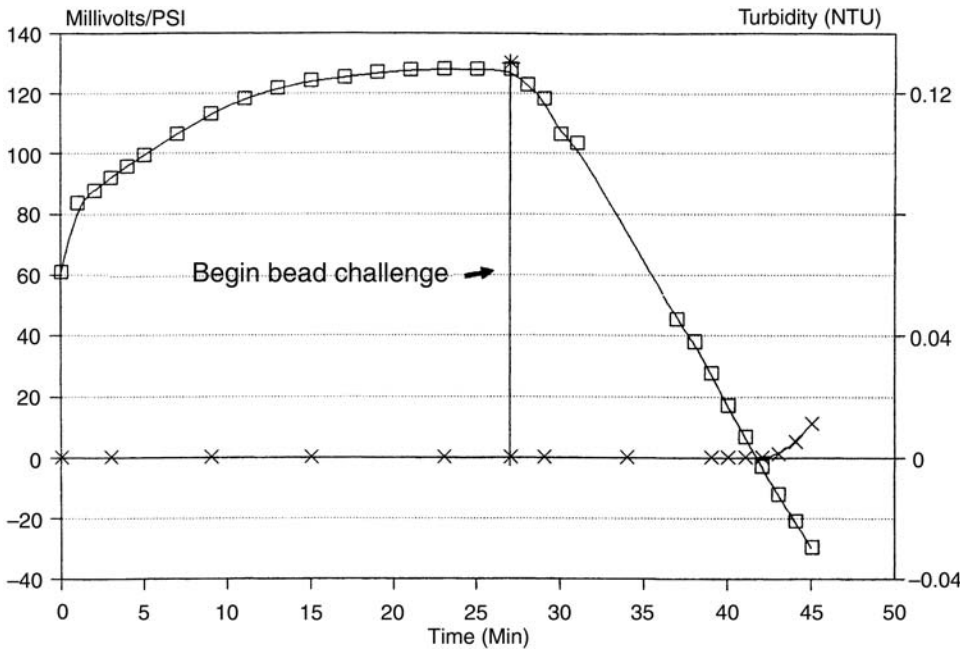


FIGURE 17 Streaming potential challenge curve. Challenged with 0.021 μm monodisperse latex beads (4.2 ppm).

Data were presented indicating that the primary mode of operation was electrostatic adsorption of the HCP by charged depth media.

An increasingly critical need for production of biologic drugs is viral clearance. There are numerous methods for achieving viral clearance including inactivation or aggregation, size exclusion retention by fine microporous membranes and adsorptive retention by chromatographic resins and filtration media. An example of adsorptive depth filter viral clearance was published by Tipton et al. (2002). The study involved investigation of viral clearance from an affinity column eluate pool. The viruses used in

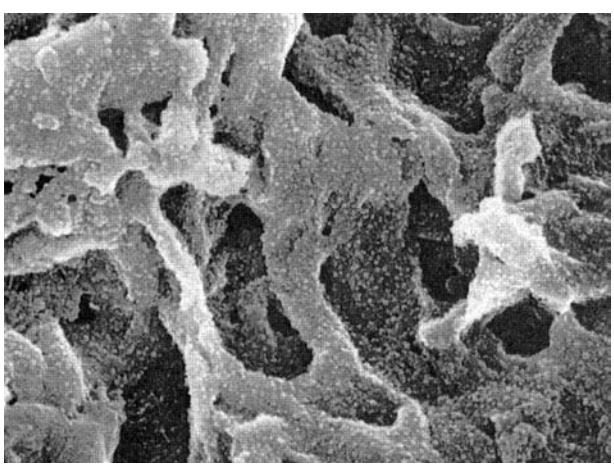


FIGURE 18 Micrograph of a 0.04 μm Zetapor membrane subjected to 0.021 μm monodisperse latex bead challenge.

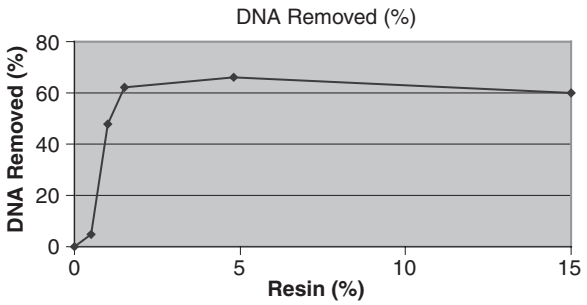


FIGURE 19 Effect of increasing cationic resin concentration on DNA retention by charge modified filter n.

the study were porcine parvovirus (PPV) with a mean diameter of 18–26 nm and xenotropic murine leukemia virus (XMuLV) with a mean diameter of 80–100 nm. Two depth filters were employed with respective tertiary and quaternary amine resin charge modification and both depth filter media having a mean pore size of 300 nm. The log reduction value (LRV) for PPV varied from 0.9 to 1.1 with the lower charge (tertiary amine) depth filter and from 1.4 to 2.0 with the stronger charged (quaternary amine) depth filter. For the slightly larger virus, XMuLV, an LRV of >4.8 was observed with both depth filter types. The slightly higher retention of the larger virus may suggest increased retention contribution due to mechanical size exclusion effects. Nonetheless, viral clearance by adsorptive depth filtration is an effective means of reducing viral contamination.

Novel techniques of separating proteins using ultrafiltration membrane, as opposed to microporous membranes or depth filters have recently been published (Ebersold and Zydney 2004; Xenopoulos et al., 2003; Mehta and Zydney, 2006). In experiments involving a process termed high performance tangential flow filtration (HPTFF), highly selective protein separations can be obtained by manipulating both membrane pore size distribution and electrostatic charge interactions. In one instance, Ebersold and Zydney (2004) demonstrated that HPTFF was able to separate protein differing by a single amino acid residue (Ebersold and Zydney, 2004). Zydney's group also published charge based protein separation involving charge modification of both the membrane and target protein. In this study a small negatively charged dye was linked to protein followed by filtration through an ultrafiltration membrane. The protein charge modification enhanced protein passage through the membrane. The technique also allows for subsequent removal of the dye charge modifier, allowing protein separation to be affected in further membrane separation processes.

Charge modification techniques can also be used to reduce membrane fouling. While microporous membranes which possess a positive zeta potential capture particles having a net negative charge, there are numerous references in the literature indicating that the addition of a negative charge to membranes, primarily ultrafiltration media and microporous media used in tangential flow applications, reduces membrane fouling by preventing non-specific binding to the membrane surface (Rao and Zydney, 2005; Mehta and Zydney, 2006; Kumar et al., 2005).

CONCLUSIONS

The current understanding of normal electrokinetic phenomena offers a particularly comprehensible or technically satisfying definition of why a given charge-modified medium functions at all, let alone so well. Understanding asbestos allowed us to create a

significant new technology. We can only believe that further understanding of the mechanisms involved will lead to the creation of both new products and new applications.

Understanding of the mechanisms involved has been, for the most part, empirically derived. Much work remains to be done in terms of optimizing both the products and the applications. This, in turn, demands a more rigorous understanding of the chemical and electrokinetic phenomena involved both in producing a surface charge modified medium and in applying such media to both existing and new applications. It is known from practical experience that charge-modified filter media provide unique separation capabilities in systems on which "normal" electrokinetic phenomena are probably not operative. The use of surface charge modified filter media to provide improved filtration performance has become an accepted technology.

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3
Filter Designs

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INTRODUCTION

Filtration is defined as the process of separation of two or more substances. This is achieved by a variety of interactions between the substance or objects to be removed and the filter. In addition the substance that is to pass through the filter must be a fluid, that is, a liquid or gas. The simplest method of filtration is to pass a solution of a solid and fluid through a porous interface so that the solid is trapped or retained, while the fluid passes through. This principle relies upon the size difference between the particles making up the fluid, and the particles making up the solid. Filtration is widely used within the biopharmaceutical industry to remove these contaminants. Microorganism removal is either required to achieve a sterile filtrate or if the pharmaceutical product is thermally sterilized to reduce the bioburden and therefore avoid elevated levels of endotoxins which is an indirect indicator of the presence of cell-debris from Gram negative organisms (ASTM F838-05).

The efficiency of a filtration process is influenced by several factors including filter design and configuration as well as a host of other factors, such as material of construction, flow requirements, sterilization, and the process of intended use. Filter manufacturers spend a significant amount of research and development efforts, time and resources to focus on these aspects while improving the filtration processes for the end users. These factors have not only influenced the design of the filters but also the functional aspects of the filters. There are many filter configurations that are available in the industry, for example, flat filters, cartridge filters, capsules, lenticular and "Pod" (another form of disposable) formats, each fulfilling a desired function, meeting an application or regulatory requirements.

FACTORS IMPACTING THE DESIGN OF FILTERS

As mentioned above, design of a filter is based on several factors including its core component—the membrane, which in itself acts as a factor that contributes to the form and function of the filter.

Membrane—Material of Construction

Filter membranes are made up of different materials of construction with specific pore ratings and chemistries and are developed by using specific manufacturing technologies.

Unique properties of materials and polymers that the membranes or filters are made of (e.g., resistance to chemicals, heat, irradiation, etc.) also influence the design of the filters. The membranes can be integral or composite in nature and can be made of cellulose acetate, cellulose mixed ester, poly(ether)sulfone (PES), nylon, polytetrafluoroethylene (PTFE), Polyvinylidene fluoride (PVDF), nitrocellulose (CN), etc. A detailed description of the properties of these membranes and their chemical and physical properties is beyond the scope of this chapter. However, it is important to understand that composition and physical properties such as membrane malleability also impact filter design, form, and function.

Filter Size—Effective Filtration Area

One of the key factors determining the design and form of filters is the ability to achieve desired (increased) effective filtration area (EFA) that will facilitate scaling and speeding up of a filtration process. Increased EFA enables further choices to be made in accordance with the volumes and types of effluents expected to be filtered under specified filtration conditions. Accordingly, the EFA can be varied or modified depending upon intended application, user needs or the desired scale of a device, for example, a cartridge may have an EFA of 0.6–2.1 m^2, the EFA of a mini cartridge may be as low as 0.05–0.2 m^2, or a small capsule may have an EFA of 0.015–0.45m^2.

FILTER FORMATS

Flat (Disc) Filters

Disc or flat filters were the first filter configuration used within the biopharmaceutical industry, mainly as 293 mm discs within large stainless steel holders. Flat membranes have proved to be excellent tools in research and development due to their size ranges of 4, 13, 25, 33, 47, 50, 90, 142, and 293 mm enabling them to be excellent choices for different types of applications (Fig. 1A). The flat discs of desired size are cut from a large membrane sheet (roll). These membrane discs are placed into a disposable plastic filter holder (e.g., Swinnex) or stainless steel holder prior to sterilization or end-use filtration. To avoid potential damage to the flat membranes, working with flat filters membranes needs careful handling and manipulation especially due to the thin and brittle nature of membranes. The membranes may potentially get wrinkled or bent during the assembly in the holders or when they are wet, causing problems during the filtration process. To minimize these issues, the flat discs available in pre-sterilized disposable devices can be used (Fig. 1B). These filters are available in different sizes and membrane pore ratings.

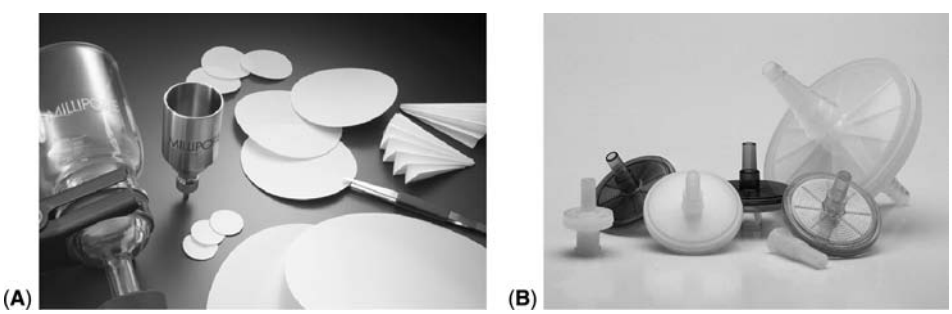


FIGURE 1 (**A**) Flat filters; (**B**) Millex family. *Source*: Courtesy of Millipore Corporation.

The most common 47 and 50 mm are utilized as microbial (analytical) assessment filters and can have different colors or colored grids printed on the membrane. The grid structure on the membrane helps counting organisms per defined filtration area and filtered volume. These filters are available in different pore size ratings (e.g., 0.1, 0.2, 0.45 and 0.8 μm) depending on the user process requirements (Carter and Levy, 1998; Baloda, 2007). They are also made of different membrane polymers such as PES, mix cellulose ester, PVDF, Nylon or cellulose nitrate. Use of different polymers provides unique performance characteristics to the device. In some cases the filter units contain a color-coded band which indicates the type of membrane that is inside the device.

Although there have been significant improvements in the performance of flat stock filter membranes leading to improved filtration efficiency, the disc filters by design are restricted by their EFA. To increase the EFA, pleated filter cartridge designs were developed to increase the filtration area without significantly increasing the footprint of the filtration system or filter holder.

Cartridge Filters

Cartridge filters are used in stainless-steel housings and provide greater EFA and filtration efficiency by virtue of their design which could include pleated membranes or stacked disc filters. The primary motivation to develop pleated membrane cartridges was the need for increased filter area that would be sufficient to secure the engineering advantages of lower applied differential pressures and larger volume flows which is particularly advantageous in more viscous liquids. This advancement in technology also had an added benefit in that less plant space was needed to be allocated for filter installations, for example, to replace a common 10-inch filter cartridge and to achieve its same EFA, fifteen 293 mm discs would be needed. Therefore the footprint of such a combination system of flat discs is by far larger than the need of 10-inch filter housing. Moreover, every disc filter would also require O-ring sealing leading to an increased set up time which may still not be a secure process. In earlier stages, the pleated filter cartridge devices contained approximately 4000 cm^2 of filtration area within the cylindrical pleat pack, which was resin bonded to the end caps (Fig. 2).

Polyester material was commonly used as pre- and support fleece. Both, the polyester and the resin used to bond the membrane to the end cap were reasons for the low chemical and thermal resistance of such filters, not to mention extractable levels, which would be unacceptable under today's standards (FDA 2004; Jornitz and Meltzer, 1998, 2000). The first membrane materials were cellulose acetate, cellulose nitrate, polyamide, and polyvinylidene fluoride. Often, these membrane materials were surface treated to achieve pleatability, wetability, and stability of the membrane, which required

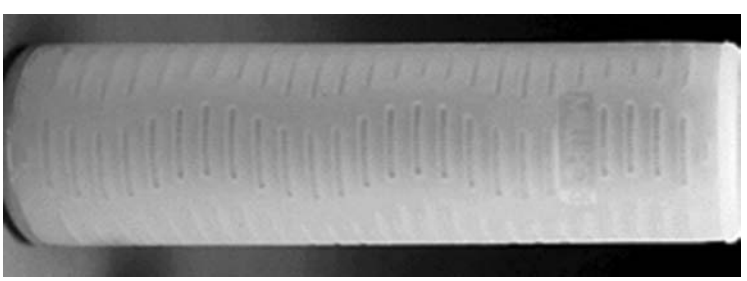


FIGURE 2 Pleated filter cartridge. *Source*: Courtesy of Millipore Corporation.

large water flush volumes before the filter could be used. Besides being a major achievement, pleating polymeric membranes has been a challenge due to the possibility of pleat breaks which is often encountered if the right pleat parameters and chemical composition have not been established. Thus, although the material of construction (core membrane) may remain the same as in case of a flat stock membrane, several other factors play a crucial role in the design and development of cartridges. These include tensile and ductile strength, elasticity, membrane thickness, type of material (polymer), membrane coating, brittleness of material used and membrane symmetry or uniformity. Each of these factors has an impact on the pleating ability of the membrane and a greater degree of knowledge and understanding of process parameters as well as the chemistry is needed in the development of a cartridge.

Accordingly, there have been significant improvements in pleating technology over the years enabling pleating of almost every type of polymer based membrane (cellulose acetate, Teflon, PES, PS, nylon, etc.). The pleats that are achieved by back-and-forth folding of the flat membrane filter upon itself lead to an increased filter surface area within a limited volume. Depending on the cartridge dimensions and size it may contain almost 5–8 ft^2(0.5–0.8 m^2) of filter surface based on membrane thickness, pre-filtration layers, and construction details. Pleated membrane cartridges are also offered in various lengths from 2 to 40 inch and EFAs, from 0.015 to 36 m^2 (Fig. 3). This range of sizes and EFAs are required for scale-up and down within the process and development steps. Yet, it is also important to understand that a pleated filter device should be able to scale-up linearly from the pre-clinical volume size to process scale (PDA Sterile Filtration Committee, 1998).

The advent of pleated filter technology has also enabled the introduction of multiple membrane characteristics in a single cartridge, for example, the ability to combine various pre-filter fleeces or membranes in front of the final filter membrane. This has also helped preclude some of the risks associated with earlier technology of stacking flat filter discs on top of each other (with an aim to increase EFA), such as insufficient sealing or unutilized EFA because of the possible air entrapment between the membranes. Pleated filters, on the other hand, already have these pre-filter combinations build into the component. The manufacturers have achieved the flexibility to combine filter combinations determined in filterability trials into a welded filter element. As a result, filtration applications can be easily optimized (Datar et al., 1992).



FIGURE 3 Different filter cartridge structures and types. *Source*: Courtesy of Millipore Corporation.

Multiple steps and components are used in the construction of a cartridge which may include lamination and pleating of membrane followed by seaming and assembly of the cartridge and final bonding of the end caps to the cartridge assembly (Fig. 4).

Elements such as 20 and 30 inch are manufactured by stacking and welding of adapters of the 10-inch elements to desired size (Fig. 4C).

Critical Elements of Cartridge Design

End Caps. The end caps of a filter cartridge are the most critical units since they are terminals for the cartridge and the pleat pack and are responsible for holding the cartridge components together. The end caps are also responsible for providing the seal between the cartridge and the O-ring recess on the cartridge-housing outlet plate. Polypropylene end caps are frequently adhered to the membrane pleat pack. The polypropylene is heated up to the melting point using various thermal methods and the pleat pack, and supporting core and sleeve are dipped into it. Various other polymers may be used, but must be selected in order to be compatible with the membrane material to insure integrity of the device.

End cap materials must also be selected to be compatible with the application streams and environments (e.g., chemical and thermal stability).

Stainless Steel Ring. A stainless steel ring is used to stabilize the cartridge orifice against steam-induced dimensional changes and as a result preserve the integrity of the O-ring seal against bypass. The use of such dimension-stabilizing rings is made in the construction of pharmaceutical-grade cartridges intended for (multiple) sterilization(s), especially when polypropylene end caps are involved. However, due to differences in the expansion rates of cartridge materials (e.g., polypropylene) during temperature changes, the stainless steel ring can also cause problems such as hairline cracks and fissures within

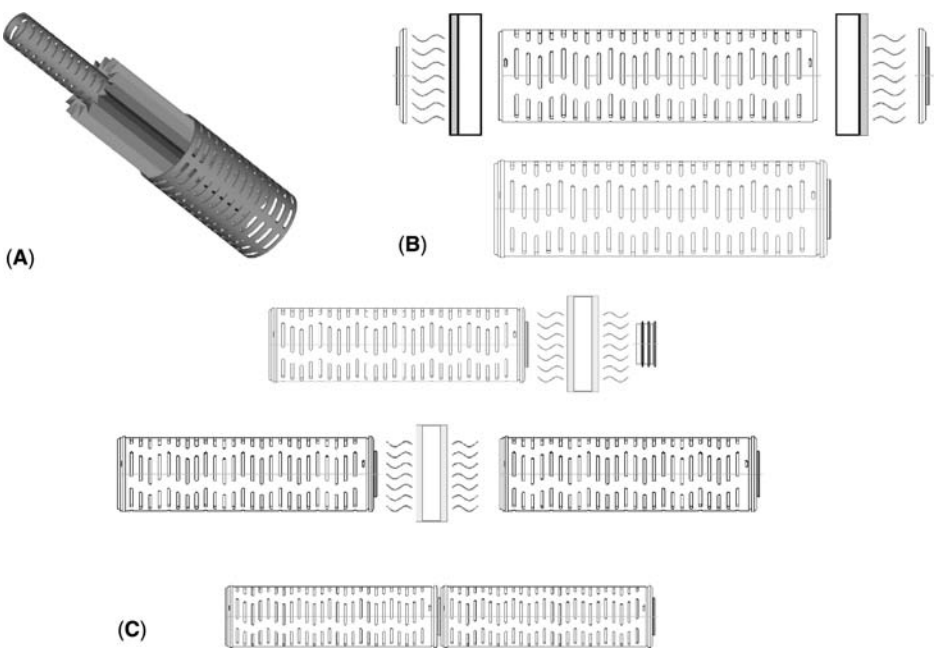


FIGURE 4 (**A**) Assembly of cartridge components; (**B**) end-capping process and final cartridge; (**C**) schematics showing bonding of two 10-inch cartridge elements for a final 20 inch product. *Source*: Courtesy of Millipore Corporation.

the adapter polymer or the welding sites leading to far greater implications such as the inability to attain proper O-ring sealing. This effect may often be seen with adapters which have not been molded from one piece. When the welding starts cracking, the liquid penetrates into the stainless steel ring cavity and expands during the next cycle of steaming (Jornitz and Meltzer, 2000). Thus, to avoid the differences in expansion of the support ring and the adapter polymer, most of the adapters are constructed with a thermally stable polymer support ring.

Outer Support Cage. The outer support cage forms the outer cylinder of the cartridge and holds the pleated internal contents together. It also acts as a backpressure guard by preventing loss of filter medium integrity as a result of fluid flowing in the opposite direction under excessive backpressure. Additionally, it facilitates easy handling of the filter cartridge during installation as well as prevents the user to come in direct contact with the pleats avoiding damage to the filter.

Outer Filter Pleated Support Layer. The outer filter pleated support layer serves as a multipurpose component. Pleating and the assembly of the membrane into cartridge form require its inclusion in the cartridge. The supportive outer pleated layer aids in protecting the filter medium throughout the cartridge pleating and assembly operation. The material also serves as a pre-filter to extend the useful service life of the final membrane that lies beneath it. Moreover, the support also maintains the structure throughout fluid processing. Without this layer, the pleats under pressure might be compressed, limiting the filter area available to the fluid processing.

Drainage or Downstream Screen. The drainage or downstream screen, similar to the outer filter pleat support, stabilizes the pleating of the pleat pack. Additionally, it keeps the filter medium pleats separated during fluid processing to assure that maximum filtration area is open for optimum flow rates and drainage of remaining filtrate, that is, reducing the dead volume or otherwise trapped fluids. The filter arrangement of the microporous membrane sandwiched between the support and drainage layers, all simultaneously pleated, is often called “the filter pack” or the “pleat pack”.

Low-melting polypropylene sealants are widely used as a sealant between the pleat pack, drainage fleeces, inner and outer cage, and the end caps. Early work on nylon cartridges attributed the generation of hydrophobic spots to this material, possibly through wicking, which impacted attempts to bubble-point the sealed cartridge. However, a less general heating of a more restricted area seems to avoid the (wicking) problem. Use of a low-melting sealant may involve some 1/2 inch of the pleat pack at each end of the filter assembly. Newer sealing techniques such as utilizing polyolefin end caps rely on fusion welding of the cap to approximately 1/8 in. of each of the pleat pack allowing the retention of valuable EFA. The aim in cartridge sealing is to utilize as few different materials as possible to minimize extractables. PTFE or PVDF microporous membranes are used because of their hydrophobicity (vent and air filters) or due to their resistance to aggressive reagents such as certain solvents and oxidizers, or hot acids (semiconductor etchants). Thermoplastic fluorinated polymers, preferably as fluorinated as possible, are used for the cartridge components and in its sealed construction. The melts supported are then usually made of a porous Teflon® material or of PVDF, as are also the remainder of the cartridge hardware from the similar polymer in its solid, impervious form.

Filter Cartridge Inner Core. The filter cartridge inner core serves as the inner hollow tube that supports the pleated pack. It confers strength to the cartridge assembly and determines the length of the final assembly of the cartridge. Moreover, the core is also the outlet port of the cartridge. The filtered fluid is guided to the outlet plate of the filter housing through the perforations of the inner core. Thus, it is essential to ensure that the cartridge core does not limit the flow except in cases of high flow applications, that is,

air filtration or water filtration with pre-filter cartridges. It must be pointed out that the flow will not drastically increase if the size of the filter is increased, for example, to a 20- or 30-inch size. The only benefit of using a larger size cartridge is the increased in service life but not an increase in flow. For this reason air filtration systems are commonly sized with 20-inch filter cartridges.

Filter Membrane. As mentioned earlier, filter membrane is the core component of the filter cartridge since it is responsible for the removal of the undesired contaminants. Liquid solutions that are to be filtered permeate into and through the filter medium and into the cartridge core, then proceed through the outlet assembly and effluent piping. Once the filter medium has become fully wetted, processing can be continued until one of several flow decay indicators signals the need for cartridge replacement, as customer preference dictates.

Cartridge designs can be manifolded and fit for the application. Not only size differences are applicable, but also cartridge adapters, that is, plug-ins, which fit into filter housings sockets and recesses (Fig. 5). A single cartridge with an end plug is used as a 10-inch filter. Otherwise it can be joined by adapters to as many 10-inch double open-end cartridges as are necessary to form the ultimate unit length desired. The filter user needs stock only three items, namely the double open-end cartridges, the adapters, and end plugs. Nevertheless, joining such 10-inch element together manually include also the risk of bypasses around the O-rings or gaskets used. Therefore these types of designs are undesirable in today's applications.

Single open-ended filter cartridges with bayonet locking are mainly used for sterilizing grade filter cartridges due to the reliability of the fit into the housing (Fig. 6). By-pass situations have to be avoided, which can only be accomplished, if the sealing between the filter cartridge and its holder is snug.

In microporous membrane applications, frequent use is made of the single open-end 10-inch cartridge, usually in T-type housings. Therefore, such a unit is manufactured with an integral end cap. Such cartridges are also constructed in 20-inch and 30-inch lengths. Attempts have been made to offer pharmaceutical manufacturers the versatility of 10-inch single and double open-end units to be assembled via adapters with O-rings. As such an arrangement increases the critical sealing area its acceptance has been limited. The more widespread use in critical pharmaceutical manufacture is of single open-end 10-, 20-, and 30-inch cartridges.

The O-ring materials used are also of critical importance, as the chemical compatibility of the O-ring material has to be determined towards the fluid to be filtered. The O-ring is the critical area of the separation between up- and down-stream side, therefore any incompatibility might be a hazard to the filtrate quality. Furthermore, in instances of multiple steam sterilization, the O-ring material has to be checked for so called heat-set. The O-ring experiences the pressure points from the housing wall and the cartridge adapter. When the temperature is elevated, as in the steaming process, the

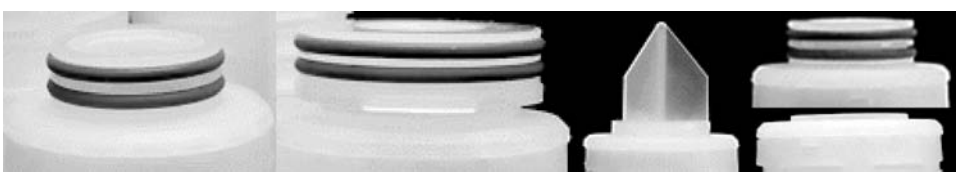


FIGURE 5 Different filter cartridge adapter types and designs. *Source*: Courtesy of Millipore Corporation.



FIGURE 6 Schematic of filter cartridge. *Source*: Courtesy of Millipore Corporation.

O-ring starts deforming at the pressure points. If the O-ring material is not flexible enough, the deformation (heat set) will be maintained. The O-ring will commonly show an oval shape. It is important that O-rings are visually inspected on a routine basis to see whether the O-ring is deformed. Any heat set might result into a by-pass situation. Although EPDM O-ring materials have shown highest heat set tendency they are very compatible to chemicals. On the other hand, silicone has commonly a high flexibility and low heat set (Meltzer and Jornitz, 1998).

In the past, the dimensions of the membrane cartridges were derived from those of the string-wound filters, roughly 10×2.5 inch. Increasing the diameters of these cartridges serves to increase their EFA (per unit number of pleats). However, most manufacturers supply cartridges with a 2.75-inch (70-mm) diameter. In general, diameters and the adapter types are standardized or similar across the industry, which creates the opportunity for the filter user to choose filters from different sources. As a result, additional capital investments into different filter housings are not necessary due to the common adapter types utilized.

The cartridge design which results in an increase in the EFA, also reflects two factors in addition to the cartridge diameter. The first factor is the diameter of the center core of the cartridge. Each pleat consists of a membrane layer (or multiple membrane layers), sandwiched between two protective layers whose presence is necessary to avoid damage to the membrane in the pleating process, and which also serves usefully in the finished cartridge as pleat separation and drainage layers. As a consequence of this sandwich construction, each pleat has a certain thickness. However, fewer of these thicknesses can be arranged around a center core of narrower diameter. Therefore, increasing the diameter of the center core increases the extent of its perimeter and the number of pleats that can surround it. This governs the number of pleats possible in the

pleat pack that can comprise the membrane cartridge, thus increasing its EFA. The use of center cores with larger diameters is particularly favorable in longer cartridges that are used under elevated applied differential pressures. The liquid flow through the microporous membrane may be so great as to find restrictions to its passage through long center cores of 'narrower' diameters. Thus, in pleated cartridge constructions intended for the high water flows of the nuclear power industry, the outer cartridge diameter may be 12 inch to accommodate a maximum number of high pleats or greater arranged around a center core dimensioned at a 10-inch diameter. The concern, exclusive of pleat heights, is to increase the service life, the throughput of the filter, by increasing its EFA (high flow rates are accommodated within the 10-inch core diameter in this application). Such restrictions to flow within cartridge center cores are generally not the concerns in critical pharmaceutical filtrations, where the applied pressure differentials are restrained in the interests of filter efficiency and longevity to yield.

The second factor governing the EFA of a cartridge, in addition to its overall diameter and center core diameter, is the pleat height. For any given pleat its height determines the surface area since larger height will result in increased surface area. The designing of a cartridge usually begins with defining of its overall outside diameter. The maximum size of the center core is accordingly determined by the maximum pleat height (e.g., 1 inch). But if the pleat height is diminished or a limiting factor for increasing the center core diameter, arranging greater overall number of pleats around the wider core may more than compensate in EFA that may potentially be lost due to pleat height diminution.

The optimum number of pleats to be arranged about a center core of a filter cartridge may reflect the filtration function or capacity for which it is intended (Jornitz and Meltzer, 2000; Meltzer, 1986). In the handling of rather clean or pre-filtered liquids (such as most pharmaceutical final filtrations), relatively few particles require removal. As a result, compaction of maximum possible number of pleats within a limited cartridge area in order to enhance the filter area may still function acceptably in these scenarios (clean streams) because the pleat separation layers will operate to make even the relatively compact surfaces individually available to the liquid being filtered. However, it is important to understand that in case of fluids with high solids, or a viscous fluid, a different situation may be encountered. The particles being removed may be large enough to bridge across a pleat resulting in the blockage of the interval between two adjacent pleat peaks. Similarly, small particles may, after their individual deposition on the filter, accumulate and grow large enough to cause bridging. Thus, irrespective of the mechanism, the bridging serves to deny the liquid being processed, access to useful flow channels bordered by membrane.

Design and manufacturing of filter cartridges also requires careful assessment of multiple parameters such as outer and inner diameter of the cartridge (Jornitz and Meltzer, 2000; Meltzer and Jornitz, 1998). An empirically developed formula that relates the outer cartridge diameter to the maximum core diameter and to the number of pleats of given height is currently used for this process. Similarly, care must be taken to protect the surface of the membrane during the pleating operation, and to avoid damage to the filter structure. Both these objectives are furthered by sandwiching the membrane between two support layers and feeding the combination to the pleater. The outlying support layers protect the membrane surfaces. Nevertheless these fleeces have to be chosen properly, for example, a fleece too coarse could press too much on the membrane at the pleating curvature and may start pressing into the membrane resulting in weakening of the membrane which might eventually prove detrimental in long-term use of the filter. Air filters are used over a long period and are subjected to multiple in-line steam sterilization.

If the membrane shows impressions due to the coarse filter fleece it may imply that the filter membrane in this area might be thinning. Multiple steam sterilization could exaggerate this thinning and flaws can develop. On the other hand a fleece, which is too soft, will not support the membrane sufficiently. Usually soft fleeces have a high fiber density and a small fiber diameter and as a result, liquid would be bound within the fiber structure. Such phenomenon needs to be avoided, for example, in air filtration, because it could cause water logging. Additionally, the sandwich in its thickness minimizes opportunities for the membrane to be too strongly compressed at the pleat. Optimally, a pleat is designed to have some radius of curvature instead of a sharp and acute angle of fold. This would prevent the membrane from being subjected to forces in excess of its mechanical properties as expressed in the magnitude of its tensile and elongation values at the pleat line. It is essential to understand that different polymeric materials have different tensile and elongation qualities and that various materials differ in their brittleness. Additionally sharp pleat edges or pleats with a high pleat density would have a gap in between the pleats, which would result into capillary activity. As a result, condensate could potentially be trapped in between the pleats and the air filter might experience water blockage, for example, in case of air filtration. Therefore, filter designs and construction require thorough investigation in development to achieve the best performance ratios. In instances the highest effective filtration are in the confined construction of a filter cartridge might not be the optimal solution, as the pleat density becomes too high. Nevertheless, EFA should also not be too low as it will influence the flow rate and total throughput. Decreasing the diameter of the center core will serve to lessen the number of pleats, although in applications which require a high flow, for example, air, the inner core becomes the flow restrictor. Therefore the inner core again needs to be optimized to the filter cartridge utilization. For example, a 28-mm core diameter will require a 40–50% higher differential pressure than a 35-mm inner core to achieve an air flow rate of 100 Ncbm. This differential pressure increase might not seem to be high, but the costs involved running such pressure difference may be substantial.

Finally, all the key factors that have been discussed above in the design and construction of a cartridge contribute to making the distinctions that define and distinguish the final cartridge product. These distinctions depend on a variety of characteristics such as membrane pore-size designation (FDA, 2004), cartridge diameter, length, type of outlet, O-ring(s) sizes, configuration of the outer end (e.g., open or closed), with or without fin, the type of O-ring or gasket seal (silicone rubber, EPDM rubber), and any nonstandard features. Manufacturer product numbers serve as shorthand substitutes for the detailed specifications.

Capsules

A capsule is a filter in a self-contained, disposable plastic housing. Commercially available disk and cartridge filters are usually disposable but their housings and holders, which are usually of metal, are permanent. However, filters encapsulated into plastic housings have been devised wherein the entire unit is disposable (Fig. 7). These devices provide significant advantages to the end user.

One of the key advantages is that many of the capsule filters are available in pre-sterilized form, for example, by gamma radiation, steam, vaporized hydrogen peroxide, or ethylene oxide. Second, they are readily available in various sizes, shapes, and formats consisting of different polymeric membranes and can be in a ready to use form on the



FIGURE 7 Different types and styles of disposable capsule filters. *Source*: Courtesy of Millipore Corporation.

shelf when needed. Although they are disposable, it does not necessarily mean that they are more expensive to use as compared to the cartridges. Thus, when making comparisons, it is essential to include both labor and material cost involving individual disposable and non-disposable assemblies. Accordingly, the installation of a single 293-mm filter disk in its housing may be more costly than the equivalent filtration area in the form of a disposable filter device especially since the use of a disposable unit entails very little setup time, without any need for additional cleanup time requirements. Thus, capsule filters eliminate the time and expense associated with assembling, cleaning, and validating stainless steel housings. Moreover, sterilization of pre-sterilized, ready to use units by the end user is also not required. Furthermore, the disposal after the single usage also eliminates risks of cross-contamination for subsequent processes.

The core components of the capsule filters are generally similar to the functional requirements (filtration) of the basic cartridge designs and most of the parameters that are essential in filter design and scale up are relevant in both the cases, for example, the need for a specific membrane polymer, flow system, function, throughput and flow rate, purpose and speed, (e.g., low protein binding), pre- or final filter, pressure rating, etc. are some of the key factors that impact the design. However, there are some key elements that are unique to capsule design. These include materials of construction, material compatibility under different conditions of use and applications, extractables and toxicity (levels) of entire device, sterilization compatibility (gamma, gassing and autoclaving, etc.). In addition to these factors, prominent features of the (functional and time tested) stainless steel housing designs also need to be incorporated in the capsule designs. Furthermore, the capsules also need to be designed in such a way that they incorporate additional features that would provide flexibility and additional benefits to the end user in their processes which may otherwise not be possible traditionally when the customer is using cartridges in stainless steel housing.

Small, but key features such as the venting of disposable filter devices require innovative approaches based on good designs and solutions. The positioning of vents (e.g., at the highest point) is one of the critical design parameters that ensures that it is both practical as well as most effective. Self-venting devices in the form of a hydrophobic membrane are another solution which permits the self-venting of air while safeguarding against the passage of liquid or contaminants (in either direction). This may be

particularly useful in water installations, where intermittent use serves repeatedly to introduce air to the system. The self-venting feature reduces maintenance and increases the system efficiency.

Designing of a disposable device leads to creativity in design and the ability to incorporate unique features such as construction of transparent shells which enable the user to observe the filtration process. Furthermore, the capsule unit could be encoded on the outside core with description of the product which ensures easy traceability, in accordance with FDA record requirements rather certain (FDA, 2004). As a result, product and batch numbers become part of the permanent operational record. Furthermore, the use of these disposables obviates the need to expense or amortize stainless steel filter holders. No capital expenditures are involved. Another advantage of capsule filter is that the user is protected from contact with the toxic product which may not be possible if a cartridge filter is used in stainless steel housing. The cartridge has to be removed from the housing at the end of the filtration run, that is, the user probably comes in contact with the filtered product remaining on the filter cartridge and housing, which may need to be avoided due to health hazards or biological activity. Disposable filters create the opportunity to replace a filter without being in contact with the product.

The disposable filter devices are available in a large variety of constructions, whether disk, multidisc, pleated cylinders of various lengths, and of different EFAs. Their expanse of filter surface runs from 4-mm discs suitable for affixing to hypodermic needles to 30-inch capsules of about 19 ft^2(1.8 m^2) (Fig. 8). The filters are made of a variety of polymeric filter materials, both hydrophilic and hydrophobic, namely, cellulose esters, polyvinylidene fluoride, polysulfone polyethersulfone, nylon, polyethylene, Teflon, etc. Their shells are composed variously of polycarbonate, polyethylene, but most often polypropylene.

The versatility of these disposable filter instruments is increased by constructions involving integral pre-filters, as in one capsule unit having approximately the EFA of a 293-mm disk. This is appropriate, as single disk filtrations most often involve applications that require the use of a pre-filter. Repetitive final filter constructions are also available in disposable unit form. These are used, for instance, in tissue culture medium filtrations where repetitive final filter arrangements are common. The increase in



FIGURE 8 Large scale disposable capsule filters. *Source*: Courtesy of Millipore Corporation.

the tailoring of disposable filter device constructions to specific application needs helps explain the mounting popularity of their usage and heightens predictions of their continuing replacement of at least part of the more conventional filter/holder market.

Because of the fragility of most membrane filters, appropriate and even extreme care is to be used in their handling. In the case of cartridge filters, this practice continues. However, the actual membrane surface of these instruments is out of reach of ordinary handling. There is, therefore far less possibility of damage to the filters. Overall, cartridges are used mostly for the more rapid flow rates and/or the large-volume filtration productions they enable, a consequence of their aggrandized EFAs. Cartridges are increasingly constructed so that their in situ sterilization can be effected by the convenient use of the steam-in-place technique.

LENTICULAR FILTERS

Lenticular filter designs are mainly used as clarifying filters. Highly adsorptive cellulosic depth filter pads are welded together in a plate format (Fig. 9). These plate formats commonly have a diameter of 12- or 16-inch and are welded together in stacks of 4–16 to create a depth filter unit.

The benefits of these depth filter materials are the tremendous dirt load capacity (total throughput). These filters are commonly used to prefilter solutions, which would blind membrane filters rapidly. Since the adsorptive depth filter material is ideal for separating colloidal substances and lipids these filters are very often found in plasma and serum applications. These filters may also be used in cell harvesting steps in downstream processing after the fermentation. When compared to the traditional technologies of centrifugation or cross-flow filtration, the combination of dirt hold capacity and reduction of the filtrates turbidity show better results than the quoted alternative technologies. Nevertheless, the selection of the separation technology of choice within the cell harvest application requires performance analysis, as the results may vary depending upon the application.

As mentioned before, the ability to scale up a filter element is essential (Meltzer, 1987). Large scale trial most often cannot be performed due to the lack of product and/or financial aspects. As a result, the filter products need to be scaled-down to perform optimization and validation trials at the lowest possible burden on product

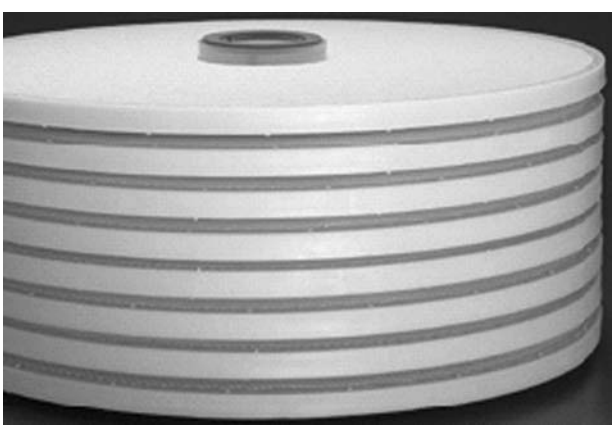


FIGURE 9 Lenticular depth filter stack design. *Source*: Courtesy of Millipore Corporation.

volume requirements. Besides the ability to scale down, it is also important to remember that the results gained in small scale trials require being linearly scalable to process scale. Any trials performed with small scale filters, which have a different design in process scale, are of no value, as the more tests are required in large scale due to the design change. For this reason, filter manufacturers have designed specific small scale devices which mirror the larger scale process filter (Fig. 10).

Since lenticular filters are also utilized in biopharmaceutical processes, these filters required to be in-line steam sterilized and fully validated. Especially leachable levels of the filters need to be low or the flush volume required to achieve regulatory requirements need to be as low as possible. Due to technological advancements over the past years, lenticular filters that have a far higher mechanical and thermal stability than in the past are now available. The construction and design of the support cages and fleeces, the welding and adapter technology has also evolved providing added design stability and safety levels. Since most of the filter pads utilized in lenticular filters are resin bonded, the filters are pre-flushed within the manufacturers production process to achieve the low leachable level required. Nevertheless, as with pleated filter devices, the leachable level should be determined within the filter users' production facility to evaluate any product or production process influences. Most of the filter manufacturers testing conditions are very specific and are commonly achieved utilizing water as a test fluid. As some products can have a different influence on the filters matrix and production parameters on the stability of the filter requires to be validated into these conditions. Again small scale device might help in this exercise.

When lenticular filter combinations are tested, the tests do not only involve the total throughput of the filter element as it is commonly the case with pleated prefilter cartridges, but an important factor is the turbidity measurement of the filtrate. The turbidity measurement creates an indication of the protective properties of the lenticular filter retention rating used and indicates how much of the contaminants are separated by the particular filter rating. Since the applications for lenticular filters vary, these filters have to undergo tests, which include the process conditions. The retentive efficiency of these filters are very much dependent on the fluid contact time within the filter matrix. The longer the contact time the better the separation of contaminants, as the main separation force of these filters is adsorptive retention. Therefore the process conditions especially pressure and flow conditions require evaluation to find the optimal total throughput combination with the lowest turbidity level within the filtrate. At the beginning of a trial the lowest possible differential pressure is used, which fulfills the flow requirements. Samples are taken in specific time intervals and the turbidity

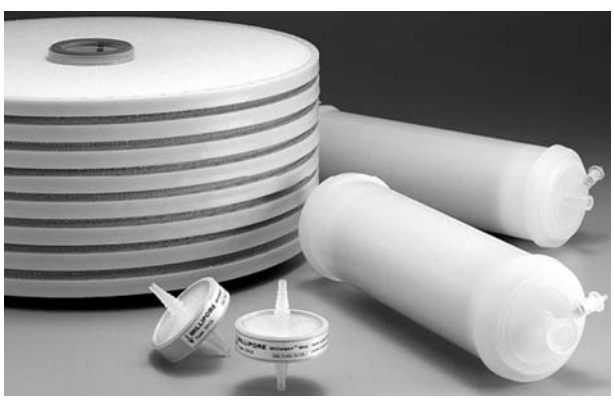


FIGURE 10 Scalable lenticular depth filter range. *Source*: Courtesy of Millipore Corporation.

measured. This gives and indication of which pressure conditions is the optimal for the filtration task, but also might show the exhaustion of the filter media, if after a certain filtered volume the turbidity of the filtrate starts rising. These tests will determine the process conditions required the filter needs to be used at. To determine which turbidity level is the optimal filtrates with specific turbidities are utilized with membrane filters, which commonly follow the lenticular prefilter. These trials will show, at which turbidity level the next membrane filter step will obtain the highest total throughput. Once the optimal process parameters are determined they are lock in the filtration protocol and the standard operating procedures.

Pod—A New Lenticular Filter Design

As discussed above, depth filters are used in pharmaceutical manufacturing to remove various particles, colloids, bacteria, and other types of cellular organisms and debris from liquid process solutions, and the cellulosic type depth filters are built on the conventional lenticular stacked-disk design. However, the ever-increasing process volumes have exposed a number of deficiencies in this filter design with respect to reliability, scalability and process economics. One particular issue which is inherent in the stacked-disk design is the possible inability to effectively integrity test the device at time of manufacture (Yavorsky, 2005).

The particular lenticular stacked-disk design described above includes a large number of mechanical compression seals between plastic "knife edges" and the filter media (seven seals per eight-cell filter). Since these seals are made by forcibly driving the knife-edge seal rings into the compressible cellulosic media then holding their position with stainless steel bands, sufficient force must be applied to close off (by compression) one or more layers of depth filter media—over-compression risks cracking the filter media. Furthermore, the need to install each lenticular style filter into a stainless steel pressure vessel and ensure proper seal compression through a spring clamp in order to conduct an integrity test is also a deterrent. This possible lack of a validated manufacturing integrity test might create a risk of inconsistent filtration performance.

These problems have been addressed in a new lenticular filter design called Pod which is a disposable depth filter that enables effective integrity testing of a depth filter. Pod design is yet another example of disposable technology and product development leading to innovative designs (Fig. 11). The Pod platform is a filter system for normal flow clarification and pre-filtration applications. Its unique design may provide greater process flexibility and productivity and also improves process speed, safety, and economics in the manufacturing of biopharmaceuticals. The Pod is built by completely encapsulating the outer perimeter of the composite depth filter media with a thermoplastic resin. Individual filter components are then welded together to form the Pod module. With the new Pod manufacturing technology, the reliability and consistent performance of depth filters can be assured. During development of the Pod design, two non-destructive integrity tests were created to validate its robust construction, with both tests intended for implementation on every device built.

The first test has been designed to check the integrity of the Pod itself against outside leaks. The Pod is pressured with dilute hydrogen gas in a specialized containment—the space around the device is sniffed with a highly sensitive hydrogen sensor to identify possible leaks. A second test is run on every filter to establish that there is an integral seal of the filter media within the Pod. The test is based on a challenge of aerosolized salt particles (NaCl) in which particle passage can be correlated to filter retention. With a mass mean diameter of 0.26 μm, NaCl particles are small enough to

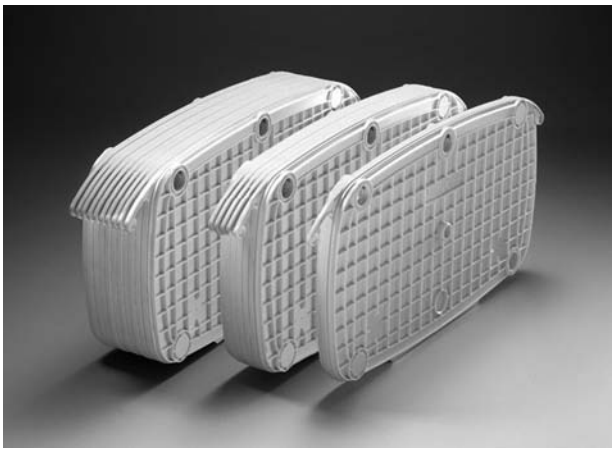


FIGURE 11 Pod lenticular filter design. *Source*: Courtesy of Millipore Corporation.

provide a critical challenge to the device seals at a retention level consistent with the filter media itself. The integrity test instrument reports the concentration of particles in the filter effluent (irrespective of size) relative to the feed or challenge concentration as a percent passage reading (Yavorsky, 2005).

Thus, the Pod filter design provides the ability to 100% integrity test a cellulosic depth filter. Furthermore, the design also provides sufficient advantages in scalability, convenience, and product yield since the Pod design consists of an expandable holder and modular filters that are designed to overcome many operational limitations of traditional stainless steel housings. It is a self-contained device and a single-use filter is housed in each Pod unit that reduces the need for clean-in-place procedures. Thus it minimizes the costs associated with water usage, cleaning, and validation. Furthermore, the design not only protects operators from exposure to process fluids, but also allows the units to be drained before change-out for lighter devices that are easier to move. In addition, the design and construction of the Pods can reduce hold-up volume from 40% to 73% over typical configurations of lenticular discs, providing an increase in product yield. Moreover, the modular Pod platform delivers process scalability and flexibility and as a result, the end users can run their system in serial or parallel configurations to meet specific process and batch size requirements of 20–12,000 l, without changing the hardware. The small footprint saves valuable floor space and the lightweight design eliminates the need for hoists or high ceiling heights. In addition, the holder is on casters for easy assembly and movement. The Pod design which is self-contained, disposable and has a modular design provides a range of improvements over the traditional depth filtration technology, including scalability, ease-of-use, and cleaning and validation requirements. This design has resulted in providing unprecedented process flexibility, productivity and economic benefits.

The Pod filter system consists of three Pod filter sizes and two expandable holders. Pods are available with 0.11, 0.55, and 1.1 m^2 filtration areas. The modular format allows multiple Pods to be combined to create the ideal solution to meet any process volume requirement. The two holders that are available, for example, the pilot scale holder which can accommodate from a single 0.11 m^2 Pod filter to five 1.1 m^2 Pods while the process scale holder expands to hold from five to thirty 1.1 m^2 Pod filters (Fig. 12). Because both holders use the same Pods, this new format offers linear scale-up and the flexibility to meet changing process needs.

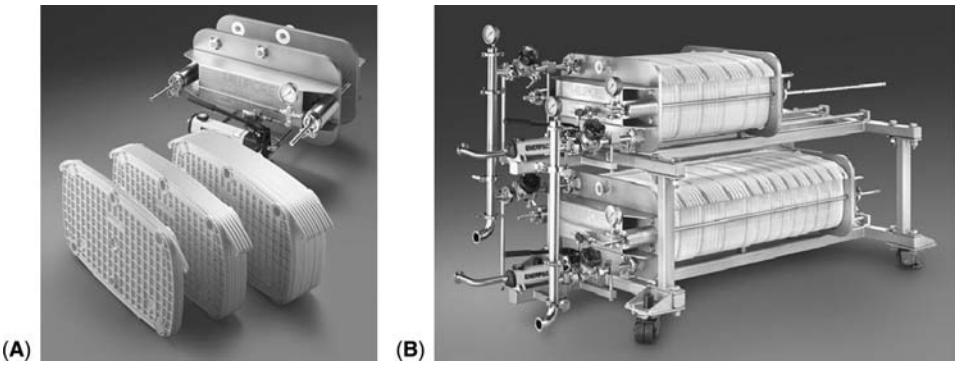


FIGURE 12 (**A**) Pilot scale Pod unit; (**B**) process scale Pod unit.

SUMMARY

The area of filter manufacturing and filter design has made significant progress over the years and this chapter highlights the evolution of this technology over the past few decades. It also demonstrates the commitment of filter manufacturers to provide unique and innovative solutions to the end users in facilitating process improvements in filtration technology. The gradual adaptation of disposable technology in pharmaceutical manufacturing has resulted in the evolution of new filter designs—a technology which is now becoming a combination of creative and artistic designs coupled with scientific advances.

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Pore Sizes and Distributions

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THE DETERMINATION OF THE PORE SIZE OF FINE FILTRATION MEDIA

Filters are used to separate particles from the fluid in which they are entrained for the following reasons or applications:

1. to separate large particles from smaller ones;
2. to recover solids from fluid streams as a product;
3. to recover solids from a fluid stream where the fluid is the product and the solid is a contaminant; and
4. special applications such as growth of bacteria.

The first of these processes is one of classification. Applications that involve the separation of aggregates into size classes, as by the use of Taylor sieves, are examples of classification processes. Of great commercial importance is the classification of fine particles used in the manufacture of magnetic devices such as tapes and computer drives. Here the particle separation must be made with very fine filters. The retention size range of these filters is very important to the process. Reverse osmosis and ultrafiltration are other instances of classification filtration.

When the removal of solids from a fluid stream is performed to recover the solid, as in filtering pigments or salts from a chemical process, total removal may not be needed. Here the medium may have a retention capability just sufficient to begin the formation of a filter cake. The cake then acts as the filter medium for the removal of the remaining solids in the stream. It is this cake that will be recovered as the product. The fluid may be disposed of or recovered and recycled in the process.

The third process is the removal of solids from a fluid stream so that the stream will be made clear, and the fluid is the product of the filtration. Beverage filtration represents this type of filtration, which is generally regarded as clarification filtration.

Membranes are generally regarded as classifying filter media. This is particularly true of uncharged media. In gas streams membranes remove particulates much smaller than their mean flow pore size (MFS) (Spurny, 1977) (Fig. 1), whereas in liquid filtration particles smaller than the MFS is retained less as the particle size decreases (Fig. 2).

Membranes that have been surface modified to produce a positive zeta potential during nitration will remove particles much smaller than the MFS. With such niters the residence time of filtration has an effect on the retention of particles smaller than the MFS. These membranes can act as clarifying filters.

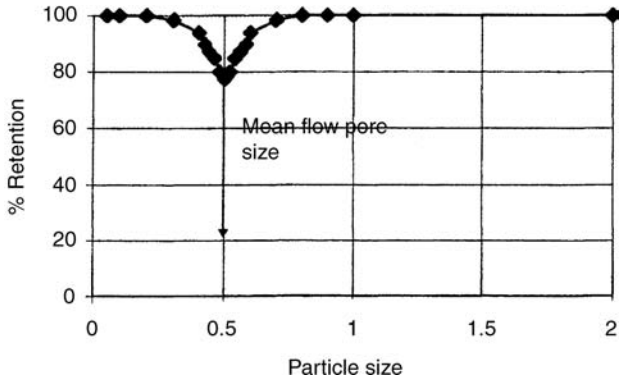


FIGURE 1 Retention in gas streams.

Filter media which are constructed of a random distribution of small particles and fibers and generally called filter sheets are typically known to be clarifying filters. Their removal capacity is limited by the interactions with particulates and the internal surface area of the medium through zeta potential, hydrophobic and hydrophilic interactions. These materials are called depth filtration media.

Filter sheets, a specific type of depth filtration medium with barely measurable bubble points, have demonstrated virtually quantitative removal of virus particles. It is almost impossible to define a pore size or particle removal size for these filter materials. Pore size measurement of these materials is irrelevant; surface area measurement, however, is of significance in establishing retention characteristics.

The measurement of the particle-retaining capacity of a filter medium should be predictable from the characteristics of the medium itself. This is more likely for classifying media, than for clarifying media. The typical Taylor sieve size can be directly measured by examining the spacing between the wires of the sieve. It is obvious that particles larger than the openings of the sieve will be retained and also obvious that particles smaller than the sieve hole size will pass through the sieve. Particles just about the same size as the holes will sometimes pass through and sometimes be trapped in the holes. These sieves have a rather sharp particle separation characteristic. With these sieves there is only one layer of sieving action to retain particles, and the hole size is very narrowly distributed. Track-etched membranes from Nuclepore also have a very narrow distribution range of pore sizes with some overlapping of holes. These filters have essentially one pore size. Separation is extremely sharp. With these filters microphotographs can be used to exactly measure the pore size.

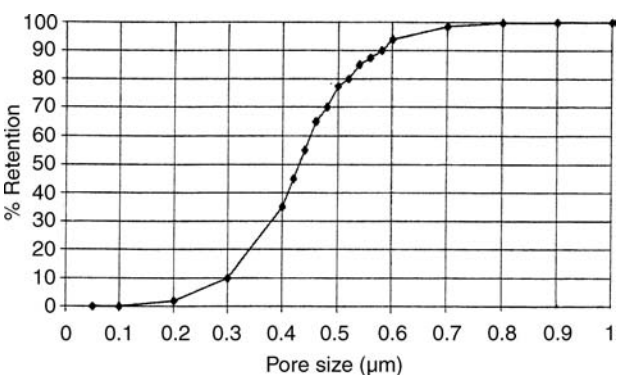


FIGURE 2 Membrane retention in liquids (noncharged media).

Filter media such as membranes formed from a phase inversion process produce a distribution of pore sizes, as is the case with most media other than woven sieves. Track-etched membranes have a narrower distribution than phase inversion membranes, but pore doubling and the etching process still result in some variation from the mean size of the filter.

With any medium there is the possibility of measuring the maximum pore size of the material by means of the bubble point. Assuming the maximum hole to be circular, the Canter equation [Eq. (1)] can be solved for the maximum-sized pore. The maximum pore is proportional to the pressure required to force air through the largest capillary or hole. With good wetting, the cosine of the wetting angle can be considered to be 1. The value of the maximum pore size obtained by using this test method has been shown to be reliable.

As the name implies, there can be only one pore of maximum size. All other pores must be smaller and not only smaller but greater in number. Finding the distribution of the pores according to their size is useful in establishing the retention and flow characteristics of the filter. Liquids flow through fine porous bodies in laminar flow (Brown, 1950) as opposed to turbulent flow. This means that the bulk flow through the capillary of the filter is inversely proportional to the viscosity of the liquid, directly proportional to the pressure differential across the filter, inversely proportional to the capillary length, and inversely proportional to the fourth power of the diameter of the capillary [Eq. (4)].

This results in the largest capillaries having the greatest proportion of total flow. Figure 3 demonstrates the amount of fluid flowing through the pores of the filter in relation to their size. The flow through the larger pores is so much greater that a numerical pore distribution is of little interest in characterizing membranes.

The flow distribution of the filter as shown in this figure (i.e., the percentage of the flow passing through each pore diameter) is significant. The pore size distribution of filters as represented by the flow distribution is what is usually meant when the "flow pore distribution" of a medium is discussed.

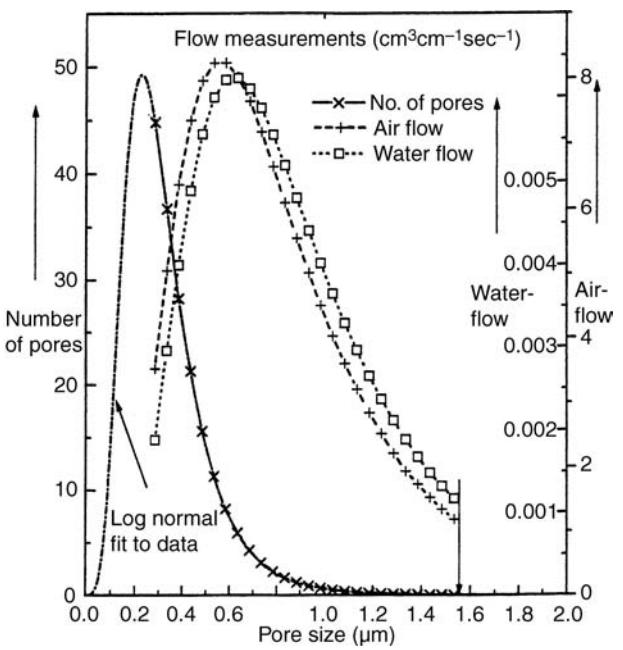


FIGURE 3 Distribution of a 0.65-μn mean flow pore filter as compared to the numerical distribution.

The distribution of flow through a filter of fine pore size (i.e., less than 1 μm) is different for liquids and gases. This is because the size of the capillaries of the filter produces a flow close to the mean free path of the gas molecules. Below 1 μm, gases flow proportionately more through a finer pore more than is predicted by the laminar flow equation. Below 0.5 μm this error is rather large. Thus, the mean flow pore size of a filter measured using gases is smaller than that which would be expected for liquids. The gas measurement gives a disproportionately higher contribution to the fine capillaries of the medium. This means that the pore size distribution of the filter should be calculated from gas flow first, and the distribution for liquids calculated from this result.

The current ASTM method does not take this factor into consideration. Media smaller than 0.2 μm will result in a considerable error in the flow distribution for liquids.

STANDARD TESTS TO CHARACTERIZE FILTER MEMBRANE MEDIA

Bubble Point

The bubble point is the measure of the maximum pore size of any filter or porous material. This is expressed by the Cantor equation, which may be derived through the analysis of the balance of forces in a capillary. Let

ΔP = the pressure differential across the filter;
D = the diameter of the capillary;
θ = the wetting angle between the fluid and the surface of the filter;
γ = the surface tension between the liquid and the solid interface material;

then

$$D = \frac{4\gamma \cos\theta}{\Delta P} \tag{1}$$

It has been demonstrated that for fluids that wet porous material well (Knight et al., 1992), the contact angle θ is 0°. This eliminates the cosine term in the equation. If the wetting fluid is water and the pressure differential is measured in bar and the pore size in micrometers, the equations reduces to

$$D = \frac{3}{\Delta P} \tag{2}$$

The bubble point is a very important test as it indicates the *maximum pore size* of the filter. It will indicate if there is an outlier pore that is larger than an expected maximum pore size. It also indicates leaks or improper sealing of a filter, be it a cartridge or "O-ring" seal of a flat membrane. In combination with other tests methods the bubble point can be used to establish the average pore size (nominal pore size) of filter media.

Water Flow Rate of Filter Media

The rate of water flow through a filter medium (Fig. 4), when determined without any pressure restrictions other than the media itself, can yield important information about the average or *hydrodynamic* pore size of the medium. With all fine pore size media the Reynolds number, Re, is usually very small, indicating laminar flow through the medium (Brown, 1950). With laminar flow, the Hagen–Poiseuille equation may be applied directly.

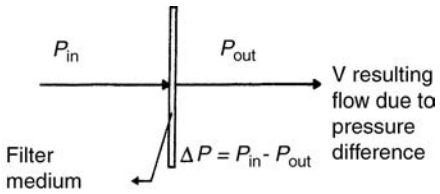


FIGURE 4 Flow of fluid through a membrane.

Assuming circular cross sections of the capillaries:

L = the capillary length, in cm;
V= the volume flow, in $cm^3\ sec^{-1}$;
η = the viscosity, in poise;
N = number of capillaries in diameter D;

Then

$$\mathrm{Re} = \frac{DV_{\rho}}{\eta} \tag{3}$$

and if the Re is less than 600, the flow is entirely laminar (Fig. 5). The flow of fluids follow streamlines ignoring the roughness and will flow around obstructions. If a particle

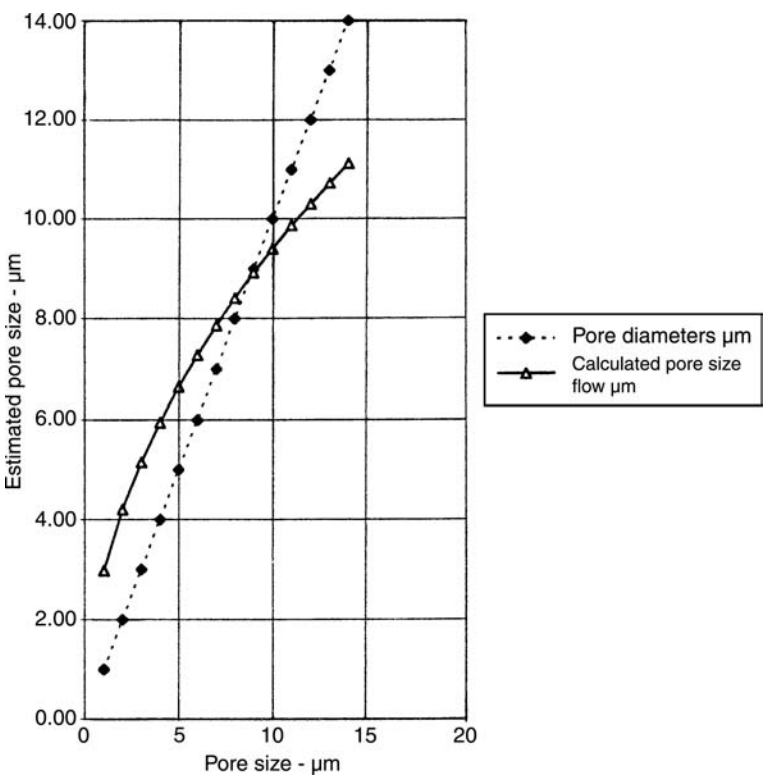


FIGURE 5 Hydrodynamic pore size estimate.

touches the wall of a capillary, it will remain there. The layer of fluid immediately next to the capillary wall is stationary.

$$V = \frac{\pi D^4 \Delta P}{128 L \eta} N \tag{4}$$

Void Volume

The water flow rate of a filter is insufficient for a determination of the average or hydrodynamic pore size of a filter medium. It is necessary to establish the area that represents the capillaries in the medium. This is determined by the void volume of the material.

Void volume V_v can be calculated from the density of the polymer or component of the medium p_m, the volume of the medium V_m, and the weight of the medium W_m.

$$V_v = \frac{W_m}{\rho_m V_m} \tag{5}$$

of the volume of an imbibed liquid V_{liq} divided by the volume of the media:

$$V_v = V_{lq} + V_m \tag{6}$$

The diameter D cannot be directly determined from Eq. (4) however, the product D^4N can be determined, as L can be assumed to be the thickness of the medium. It is usually possible to determine the void volume of the medium from density determinations. With this information, the number of pores can be calculated as a function of pore size.

If V_v is the void volume of the medium and A is the area of the filter in square centimeters, then

$$N = \frac{4A}{\pi D^2} V_v \tag{7}$$

Solving Equations, (4) and (7) simultaneously (which can be done graphically), the hydrodynamic pore size of the medium is determined. A medium that has a skin as the ultrafilter medium requires that the thickness of the skin be used in this determination instead of the thickness of the structure. If this is not done, the pore size can be calculated as being larger than the maximum pore size. The following example illustrates the procedure.

The medium is a porous carbon block filter, as represented in Figure 6. An estimate of the average pore size was needed to see whether this material could retain a particular particle size. First, the bubble point of the carbon block was measured using water. The value of pressure at the bubble point was 3.61 psi or 0.25 bar. This, from Equation (2), gives a maximum pore size of 12.05 μm. Second, the axial water flow rate of the block was measured. Next, the void volume of the block was determined. The volume of this block is 64 cm^2. Carbon's specific gravity is 1.68 g cm^3, which yields a carbon volume of 23.6 cm^3. When the carbon block was completely filled with water, which was accomplished by immersing the block in water in a vacuum, a water volume of 40.4 cm^3 was measured. This calculates to a void volume of 63.2%.

This means that if this block were composed of capillaries uniformly distributed throughout the block, the area of these capillaries must equal 8 cm^2. From the flow test, a flow of 6.67 cm^3 sec^{-1} was found with a capillary length (block length) of 4.9 cm at a

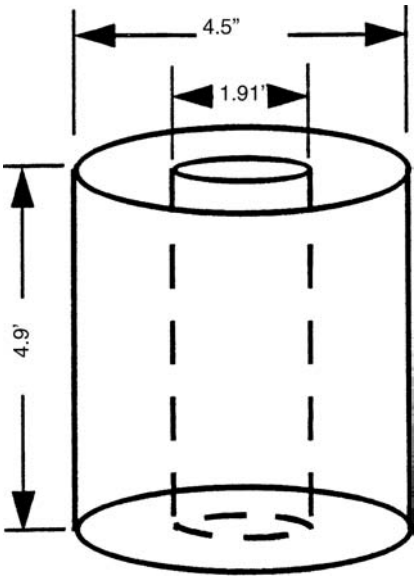


FIGURE 6 Carbon block filter dimensions.

pressure differential of 1.66 bar. The simultaneous solution of Eqs. (3) and (5), which is graphically solved in Figure 5, is about 8.5 μm. The calculated hydrodynamic pore size of this filter is smaller than the maximum pore size, as determined by bubble point measurement. If this was reversed, it would indicate that the block had a nonuniform structure such as that produced by a dense surface skin. This means that the assumption that the capillary length is equal to the thickness of the medium is incorrect. In this block the flow radially through the filter resulted in the same pore size being calculated. There was obviously no skin or surface densification. When there is surface densification, there can be a discrepancy between the maximum pore size and the calculated hydrodynamic pore size. The value of L must be adjusted to take the densification into account. Sometimes it is difficult to make a conclusion that can be supported by data in the determination of L for a dense surface.

In membrane filters this can be very important in the determination of pore size and retention characteristics. It is possible to measure the pore size distribution independently of surface densification. A rigorous analysis of this process requires the solution of the flow equation for a wetted filter medium. For membrane filters in the pore size range of about 0.1–3 μm, the following equation has been developed:

$$PV = \frac{\pi D^4 \Delta P \bar{P}}{128 \eta L} = nRT \tag{8}$$

This represents the viscous portion of the gas flow. An additional term must be added to this equation when the Knudsen number, Kn, is greater than zero. The flow equation for small capillaries is (Carman, 1956):

$$PV = \frac{pD^4 DP\bar{P}}{128hL}\left[1 + 4\ \mathrm{Kn}\left(\frac{2}{f} - 1\right)\right] \tag{9}$$

Kn is the Knudsen number, the ratio of the mean free path of the gas molecules to the diameter of the capillary. In membrane filters the value of Kn cannot be ignored. In this equation f is a reflection factor. This factor is absent from the viscous flow equation because the molecules never reach the surface of the capillary, because a layer of

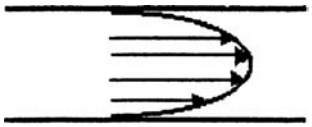


FIGURE 7 Vector representation of laminar flow in a capillary.

motionless fluid lies on this surface. The profile of the velocity within the capillary will have the shape shown in Figure 7. The arrows are the varying velocity vectors and the curve is the profile of these vectors. When the Kn number becomes significant, the velocity at the surface of the capillary becomes greater than zero and the molecules can interact with this surface. Thus, there can be some reflection from rough spots in the surface.

Membrane filters are not capillaries but are composed of cells and openings in the cells, and thus there will be ample opportunity for reflection from the internal surfaces of the membrane. Streamlines will no longer completely surround the surfaces of the membrane structure preventing any reflection. The higher the Kn number, the greater is the proportion of reflected molecules (see Fig. 8) of the fluid stream (Tsien, 1946; Carman, 1956).

The value of f for a porous body is usually constant over a wide range of flows and pressures. This has been demonstrated in direct measurements of the flow of gases through membrane filters (Badenhop, 1983).

Substituting the specific gas characteristics for the value of the Kn number into Equation (7) (Badenhop, 1983:41–55):

$$\mathrm{Kn} = \frac{\mathrm{Kn}}{\Delta P P} \tag{10}$$

and rearranging terms and dividing through the ΔP

$$\frac{PV}{\Delta P} = \bar{P}\frac{\pi D^4}{128\ \mu L} + \frac{\pi D^3 4\mathrm{Kn}}{128\ \mu L}\left(\frac{2}{f} - 1\right) \tag{11}$$

If the value of f is constant, the preceding equation is linear when $PV/\Delta P$ is plotted against P. The graph of the flow of hydrogen and nitrogen through a 0.1 μm filter (Fig. 9) shows the linearity of the relation between $PV/\Delta P$ and P, which proves that the value of f is constant through this membrane (under the conditions of the test). In addition, the intercept of this function does not pass through the origin as it would if the Hagen–Poiseuille law were followed.

Thus, a significant increase in the expected flow occurs through the gas slip effect. If the calculations used to determine a pore distribution of hydrodynamic pore size uses the Hagen–Poiseuille law, in this calculation, without the consideration of gas slip, errors occur. The smaller the capillaries, the greater the error. Note also that this linearity verifies Equation (11). It correctly represents the flow of gases through membrane filters.

$$PV_{\mathrm{vk_i}} = \frac{\pi \Delta P_\mathrm{i}}{128\ \eta L}\left\{\bar{P}_\mathrm{i}\int_{D_\mathrm{i}}^{D_\mathrm{Bp}} f(D)D^4 dD + 4\ \mathrm{Kn}\int_{D_\mathrm{i}}^{D_\mathrm{Bp}} f(D)D^3 dD\right\} \tag{12}$$



FIGURE 8 Partial molecular flow in a small capillary flow extends to the wall of the capillary, causing reflection due to roughness.

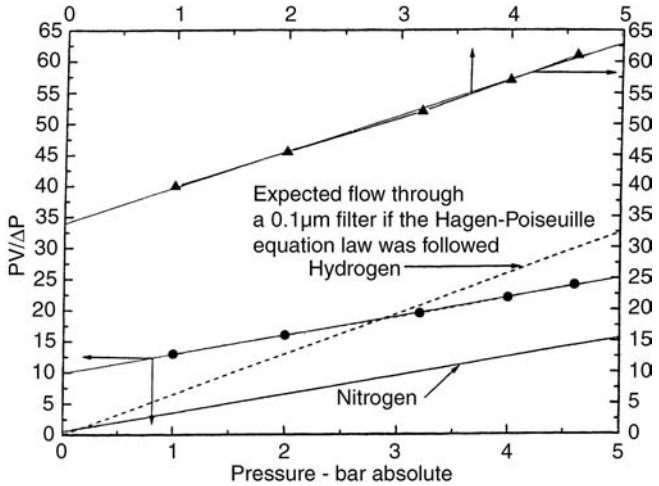


FIGURE 9 The reflection factor f is constant over the test range.

where

$f(D)$ = the pore distribution function;
D_{BP} = the maximum pore size (bubble point);
D_i = a point on the wet flow curve between the bubble point and the smallest possible pore size.

Each point D_i, on the wet flow curve is represented by Equation (12). From this equation the value of the distribution function $f(D)$ can be determined. Note that integrating this equation (assuming the distribution function $f(D)$ were known) would yield the dry flow of the membrane.

The procedure for development of the distribution function is one of careful measurement of the wet now versus pore size at ever-increasing pressure in steps of constant pore size. At each step, beginning with the bubble point, where the flow is just >0, a step size is chosen small enough to generate a reasonable function but large enough to control pressure differentials exactly. It is also of critical importance that the wetting fluid be nonvolatile so that the only increase in flow, with each increase in pressure, is due to pores previously open flowing at a higher AP—and new pores being opened as the bubble point of these small capillaries are exceeded. The flow must be measured after sufficient time to assure the flow through the newly open capillaries has reached the maximum. The wetting fluid must have time to be blown out of the capillary.

It sometimes requires up to 5 min to achieve constant flow. The number of pores is then calculated from the effect of the increase in pressure after taking into consideration the added flow from pressure increase from the previous step using Equation (13), the general equation of flow (Badenhop, 1983):

$$PV = \frac{\pi D^4 \bar{P} \Delta P}{128\ \eta L}\left\{1 + \frac{4\ \mathrm{Kn}}{DP}\left(\frac{2}{f} - 1\right)\right\}N \tag{13}$$

For the normal pore distribution, as shown in Figure 10, the flow pore distribution has been calculated using the preceding equation. Figure 10 is the normal distribution about a numerical mean pore of 0.6 μm and a maximum pore size of 1.2 μm. The resulting calculation of the flow pore distribution is shown in Figure 11. Real membrane filters tend to be log normally distributed. An actual example of the pore distribution from

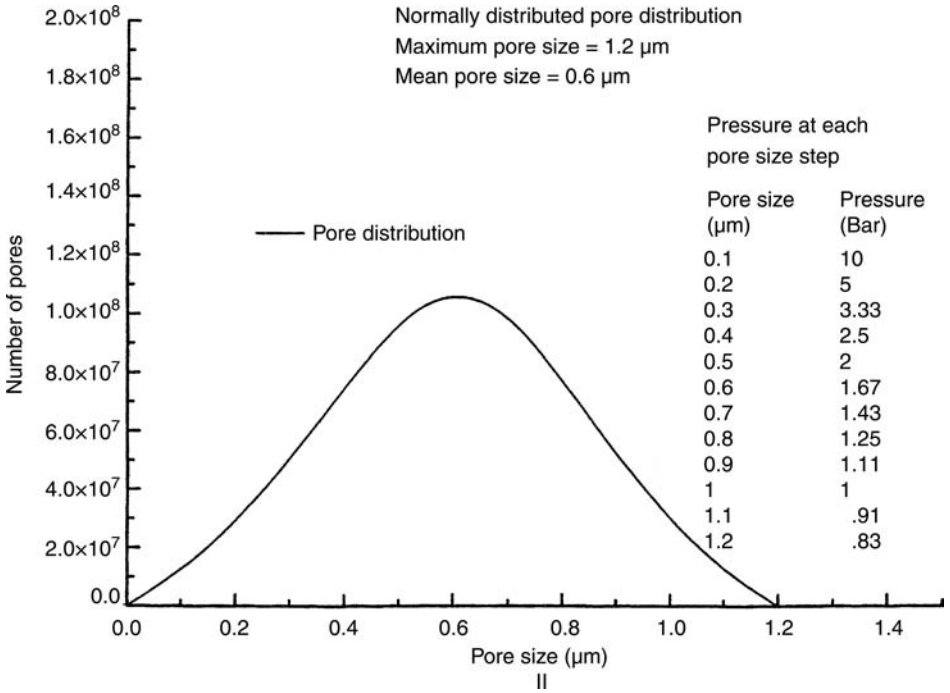


FIGURE 10 Numerical pore distribution, normally distributed.

measured data is shown in Figure 12. Note that the distribution for air is slightly different from the distribution for water.

EVALUATION OF THE INTEGRITY OF LARGE-MEMBRANE FORMATS

The measurement of the bubble point of membranes in small formats presents no difficult as the initiation of flow may be either directly observed or measured with sensitive instruments. As the area of the membrane increases, the difficulty of determining the bubble point increases because of the parallel flow of gas from diffusion through the wet membrane. With small formats this diffusion is almost immeasurable. With larger formats diffusion flow becomes significant, especially at the high pressures needed to assure integrity of cartridges with very small pore size membranes.

The diffusion of gas through the membrane is not at all influenced by its pore size. Pick's law, which describes the diffusion of gases, states that the diffusion is proportional to the pressure across the membrane and inversely proportional to the thickness of the wetted film of the membrane. The rate of diffusion is also related to the solubility of the gas in the testing fluid. CO_2, for example, has a higher diffusivity through water wet membranes than does air. SF_6 has a lower diffusivity through water wet membrane than does air.

Membrane cartridges are normally tested with air, and the diffusion rate at a fixed pressure is used to indicate the integrity of the membrane composite. As this test measures the diffusion of gas through a wet film, it is not measuring the pore size of the membrane. For this reason the test pressure used in establishing the integrity of composites is usually set some point below the bubble point of the media (to avoid

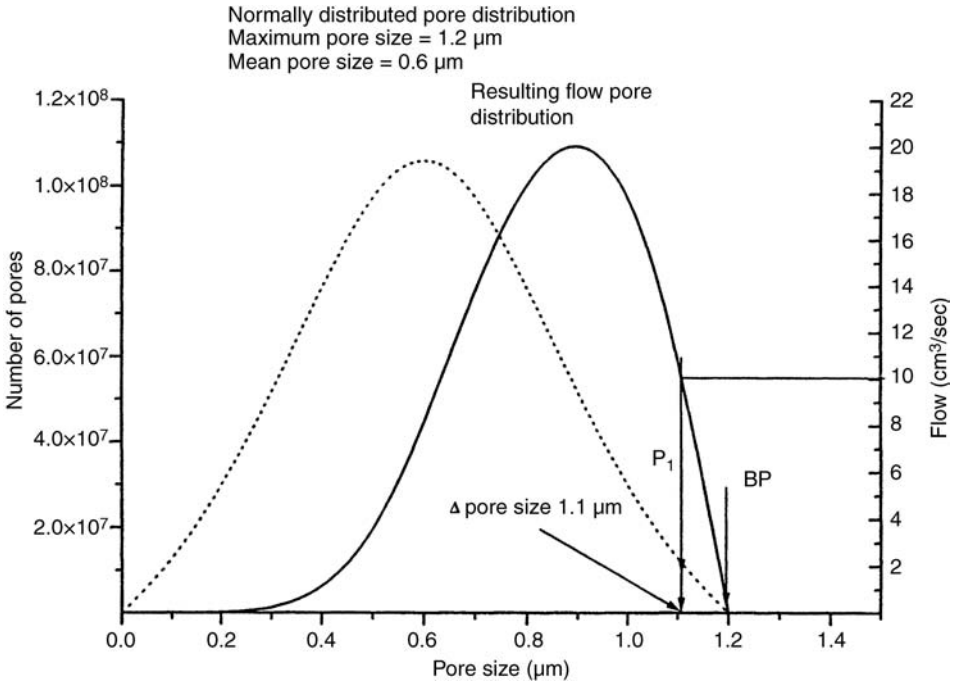


FIGURE 11 Normally distributed numerical pore distribution showing the resulting flow pore distribution.

exceeding the bubble point, which would result in very high flow values), and high enough not to be below the bubble point of the next larger grade of membrane. Thus, the test is performed, for example, for a 0.2-µm cartridge at a pressure higher than a 0.45-µm membrane's bubble point and lower than the 0.2-µm bubble point. It is generally set at 80% of the bubble point of the medium used to produce the composite.

The level of diffusion flow will increase as the pressure increases and finer pore sizes are evaluated. It is, however, very difficult to distinguish between a level of diffusion flow and small defects in the cartridge. This is of critical importance when determining the integrity of pharmaceutical membrane cartridges, as a single defect can result in bacteria passing through the structure (Badenhop, 1990).

Attempts at developing testing procedures that would distinguish between diffusion and bulk flow have been undertaken since 1980 at AMF laboratories (Badenhop, 1990), using a procedure that utilizes the time delay in the establishment of diffusion of SF_6 gas from the almost instantaneous development of bulk flow.

Companies other than the membrane producers have begun developing testing equipment for the measurement of porous bodies (Knight and Badenhop, 1990). (These are well known in the industry and include Sartochek from Sartorius, Pall's Palltronic, etc.) Coulter has marketed a porometer for the measurement of pore size distribution, as has Porous Materials Incorporated (PMI). PMI has been developing integrity testing devices utilizing mass flow meters in their design. In addition, they have been able to build very repeatable equipment by using oversampling measurement techniques which hold pressure constant until flow has stabilized. This oversampling is of particular help in establishing the true diffusion/bulk flow characteristics of membrane cartridges. The PMI system is capable of establishing the distribution of the pore structure at the bubble point of cartridges because of the accuracy of the instrumentation.

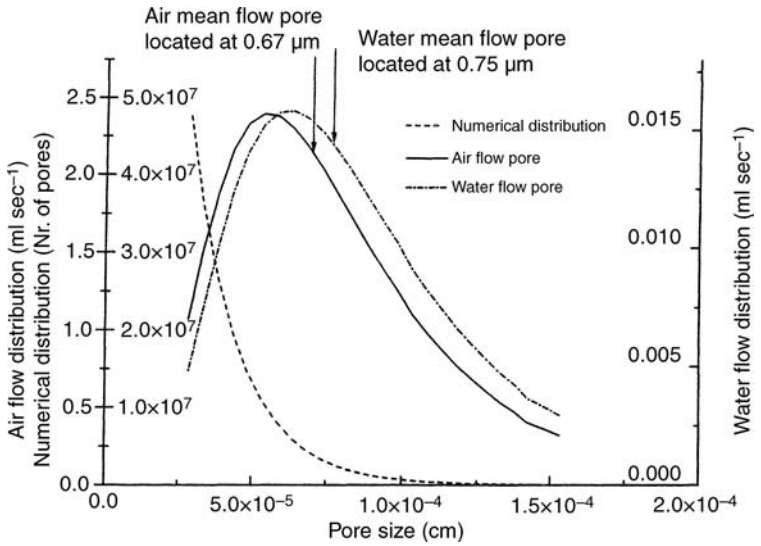


FIGURE 12 Distribution of a 0.65-µm membrane.

In order to refine the measurement of cartridges, an improvement in the signal-to-noise ratio of the measurements is of considerable advantage in generating definitive data. This is critical in producing truly meaningful results that distinguish between bulk flow and diffusion. It is possible to develop such a system.

The foundation of the mechanical requirements of a highly precise system for reliable integrity testing is already available in the PMI equipment and software. The second step in the development of a high-sensitivity measurement system is the incorporation of gases other than air in the diffusion testing. With the use of other gases, the sensitivity of the mass flow meters improves significantly. Using SF_6, as an example, increases the sensitivity of the meters by a factor of 4.

Of particular interest are gases that are nontoxic, less soluble in water, and lower in viscosity than air. The Freons have this characteristic but are expensive and environmentally unsound. SF_5 is a nontoxic gas that we have studied in the past. It is nontoxic, the mass flow meters are four times more sensitive to SF_6 than to air, and it is lower in viscosity than air. The viscosity of air is 0.00186 cps and SF_S is 0.00152 cps, an improvement of 22.37%. This has the net effect of enhancing the differentiation between bulk and diffusion flow measurements. SF_6 also is one-fourth less soluble in water than air. Thus, there is a bulk flow-to-diffusion improvement of almost five times that of air.

This difference is particularly noticeable when the output voltage off the mass flow meters using SF_6 are plotted against the output voltage of the mass flow meters flowing air (see Fig. 13).

The flow of gas through small pores (larger than 1 µm) is approximated by the viscous flow equation:

$$V = \frac{\pi D^4 \Delta P}{128\ \mu L} N \tag{4}$$

From the Cantor equation, which relates the bubble point of the membrane to the pore size, the relation between bubble point and maximum pore size is given:

$$D = \frac{4\gamma \cos\theta}{\Delta P} \tag{1}$$

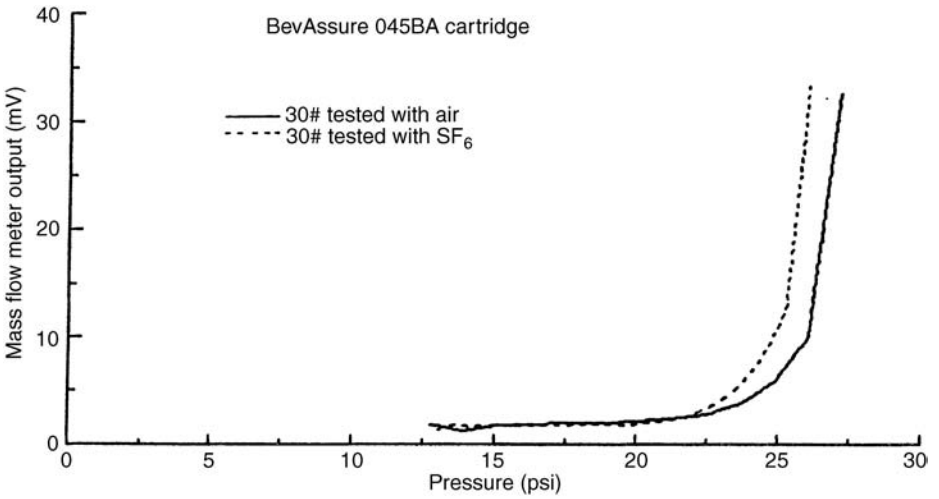


FIGURE 13 Output voltage off mass flow meters using SF_6 versus air.

Since the wetting angle has been shown to be 0° (Knight et al., 1992) this reduces for water and when the pressure difference (in bar) and diameter (in μm) to

$$D = \frac{2.88}{\Delta P}$$

With the pressure in psi the relation is

$$D = \frac{41.76}{\Delta P}$$

Using these relationships the number of pores in bulk flow can be calculated from the total of diffusion flow and bulk flow by measuring the flow above the linear Pick's law values.

TABLE 1 Gas Flow Values for Flow of Air Through Defects of Various Sizes

	Flow of air through defect size (ml/min)				
Test pressure bar gauge	10 μm	8 μm	6 μm	4 μm	2 μm
1	0.52308	0.22302	0.07200	0.01433	
1.5	0.85020	0.37626	0.12444	0.02503	0.00157
2	1.18560	0.54702	0.18630	0.03799	0.00239
2.5	1.53960	0.72900	0.25626	0.05315	0.00336
3	*1.61700*	0.91980	0.33282	0.07044	0.00448
3.5		*1.03500*	0.41478	0.08982	0.00575
4			0.50058	0.11088	0.00716
4.5			*0.58200*	0.13386	0.00872
5				0.15858	0.01044
5.5					

Italic numbers indicate choke flow.

TABLE 2 Gas Flow Values for Flow of SF_6 Through Indicated Defect of Various Sizes

	Flow of SF_6 through defect size (ml/min)				
Test pressure bar	10 μm	8 μm	6 μm	4 μm	2 μm
1	0.00791	0.00380	0.00134	0.00028	
1.5	0.01199	0.00600	0.00223	0.00048	3.07E-05
2	*0.01384*	0.00843	0.00325	0.00072	4.68E-05
2.5		0.00957	0.00437	0.00100	6-58E-05
3		*0.01023*	0.00561	0.00132	8.76E-05
3.5			0.00167	0.00167	1.12E-04
4			*0.0064*	0.0021	1.40E-04
4.5				0.0024	1.70E-04
5				0.0029	2.04E-04
5.5				*0.0033*	2.40E-04

Italic numbers indicate choke flow.

The viscous flow equation holds for gases only when the velocity of flow is less than Mach number (Ma) 0.5. For membrane-sized pores this is not a consideration; however for defects, which are much larger than the membrane pore sizes, the effect of the Ma should be evaluated. The gas flow values for assumed defect sizes shown in Tables 1 and 2 have taken the compressibility of the gases into consideration. Note that once $Ma = 1$ no increase in flow through the pore is possible with a decrease in downstream pressure. Increasing upstream pressure increases flow due to density change only. This further complicates the flow calculations.

For SF_6 the molecular weight is 146 and the higher density results in a much lower speed of sound, which limits the flow at higher pressures. This, in effect, makes the use of the SF_6 more sensitive in the lower pore size areas for defects in the bubble point range. The Ma number exceeds 0.5 even at 0.5 bar pressure, indicating compressible flow.

The apparent accuracy of the measurements made with the PMI equipment is in the range of ± 0.0125 ml of SF_6. Assuming that it is possible to read to ± 0.02 ml of SF_6, with careful testing, the character of the defects can be discernible. This level of accuracy is not possible with air and is due to the enhanced sensitivity of the meters using SF_6.

The accuracy of the PMI equipment is largely due to the oversampling methods of developing the data. The equipment raises the pressure to a desired value and waits until the flow gauges give a constant reading. This allows time for pores to open because their bubble point are exceeded and to reach a steady flow rate. Once the steady flow is established and the measurement is recorded, the pressure is increased to the next level.

This oversampling is particularly critical in determining the bubble point and mean pore of the filter. Failure to wait for the flow to come to a constant rate will result in inaccurate and nonrepeatable test results. There can be a substantial time delay in the establishment of the mean pore that will require the instrument to wait for 5 min or longer at the pressure near the mean. The PMI equipment is capable of adjusting for this effect.

The incorporation of a halogen-containing gas, such as SF_6, in the testing allows the use of Bragg Curve Detector (BCD) detectors that can significantly increase sensitivity in determining the point at which bulk flow begins (detection of the bubble point).

Using a mixture of SF_6 and air in diffusion testing will take advantage of the wet membrane's gas separation capability. The mixture will have a different composition when passing through the filter by a diffusion mechanism than when passing through the

filter by bulk flow. The difference in the solubility of air and SF_6 is about 4 to 1; thus most of the SF_6 will be retained as the mixture diffuses through the filter. The BCD detector is extremely sensitive to halogen-containing gases and the change in composition because of:

> Bulk flow containing four times the SF_S from bulk flow rather than diffusion the lower viscosity of SF_6, which further enhances the sensitivity of the measurement.

There is a considerable cost advantage in using a mixture of SF_6 rather than pure SF_6, and the detectors will be able to sample the total gas mixture when the correct composition is chosen. This latter point was a significant problem in the earlier work at AMF, complicating the sampling of the gases.

A definitive single-point measurement is possible with this system. It is only necessary to measure the composition of the gas to indicate whether bulk flow existed at the test pressure. As bulk flow occurs, there is an increase in the flow of the gas through the cartridge. This composition change of the mixture of the gases will be identified by the BCD detector. This is a far more sensitive measurement than a flow measurement made by mass flow meters.

In addition, when pressure is applied quickly, bulk flow occurs almost instantaneously, whereas diffusion flow requires saturating the water film with the gas mixture. This phenomenon has been described by Brown (1950). This time delay allows for true detection of leaks in a large filter system.

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5

Concerning Mechanisms of Particle Removal by Filters

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INTRODUCTION

That a particle larger than an opening cannot possibly fit through it without being distorted has been so commonly experienced that it is regarded as being self-evident. It is on the basis of this axiomatic understanding that the mechanism for the separation of particles from fluids by filter action is universally comprehended. Particles suspended in fluids are restrained by their size from negotiating the pores of the filter while the suspending liquid flows through unimpeded at a rate that is most usually a direct function of the applied differential pressure.

The pores, being tortuous, are longer than the membrane's thickness which for most microporous membranes is about 15 mils. The operative particle retention phenomenon is described variously as "sieve retention" or "size exclusion," or some like descriptive term. So apt is this portrayal that assaying the pore size is attempted from measurements of the rate of fluid flow through an orifice. Likewise, the arrest of a particle by a filter pore serves to quantify the size of one relative to the size of the other. In its simplest form, this relationship assumes that both the pores and particles are each monosized. The particle shape and its orientation relative to that of the pore is important. It is conveniently assumed that the pores are circular in diameter and that the particles are spherical in shape. These oversimplified assumptions are rarely in accord with reality. Where non-spherical particles are involved, the frame-of-reference method of determining the average projected area of the particle is by particle counters that program the projected area and deduce from it the diameter of a circle of equal area. Other type counters disclose either the average projected area of a tumbling particle, or its volume. Correction factors must be developed for use with either type particle counter (Johnston and Swanson, 1982).

Actually, not too much is known about the pore structures, nor is enough known about the shapes of the particles undergoing restraint by the filter. The pore shapes are described as being tortuous. Usually, the particles being arrested are neither spherical nor monosized. The filter pores, too, are not all of one diameter, and probably no pore, being sinuous, is uniform in diameter. Each pore is likely to periodically feature constricted areas, whether at its entrance at the filter surface or within its interior. It is at these smallest diameters that the particles undergo size exclusion.

The pore structures of the microporous membranes represent the paths of least resistance to fluid flows. They are not firmly established integral pathways extending through the filter from one surface to the other as in a sieve. Excepting track-etched membranes, they are assemblies of spatial vacancies in the filter matrix that, depending upon filtration conditions such as the degree of particle loading and viscosity, may periodically become blocked by particle accumulations. At such times, an ad hoc synthesis of available openings forms a new path of least resistance through the filter.

THE SIEVE RETENTION MECHANISM

The sieving mechanism is perhaps the most familiar manifestation of filter action. The particle is retained because it is too large to fit through the filter mesh or pores. For monosized particles and a filter of identically sized pores, the retention is independent of the number of particles or pores. It is independent of the filtration conditions. For example, the differential pressure motivating the fluid's flow, unless it is high enough to deform the suspended particle, does not affect the particle removal. The nature (polymeric) of the filter, as also that of the particle, are of no concern unless the physicochemistry of the fluid vehicle reduces the particle size or enlarges the pore size. Each of these is a real possibility under certain conditions. Filter efficiency is then threatened.

Filter action becomes complicated when there is a spread to the size particles and pores. In the real world both the particle and pore sizes are characterized by size distributions. The selection of the filter is made largely in the expectation that its pores are sufficiently small to retain the particles while generous enough in size to minimally restraining the rate of flow. At best, making the choice depends upon some relevant experience. If the basis of the filter selection is rendered inappropriate by changes in size of either pore or particle, a probability factor then governs the filter efficiency. It depends upon the likelihood of small particle/large pore encounters; which in turn depends upon the ratio of smaller particles (organisms) to enlarged pores. In essence, the size alterations create a size distribution situation.

The particle shape, especially in conjunction with the rate of flow as produced by a pressure differential, may well have an effect on retention. An elongated, slim particle may more likely be oriented longitudinally to the direction of flow by increasingly higher flow rates. Its likelihood of being retained would depend upon the size and shape of the pore and upon the flow velocity. It could tend to escape capture were it directed to the brief (1 mil), straight-through columnar pores of a track-etched membrane. The shapes of particles and of pores are rarely known. Approximations of how particles and pores interact with regard to their sizes are usually based on assumptions that particles are spherical and pores are circular.

Statements are made on occasion to the effect that membrane filters are absolute. The term "absolute filter" signifies an unqualified success to its utilization; a freedom of its performance from dependency on particular conditions. Even in situations where there are particle and pore size distributions, as long as the smallest particle is larger than the largest pore, the filtration is absolute. But *only* in that circumstance may the filter be so characterized. In a filtration operation, either the particle or pore size distribution is seldom known. The term "absolute filter" is a marketing term. It does not belong in the technical literature. Absoluteness is a relationship of a filter's pores to a collection of confronting particles. It is not a filter property.

The sieve retention mechanism is easily understood; the particles larger than the filter pores they encounter are restrained by size exclusion; the smaller particles and fluid are not so retained. Its effectiveness depends solely upon the numbers and sizes of the particles and pores, and to that extent may be probabilistic, depending upon what size particle meets which size pore.

The sieve retention mechanism is relied upon in a variety of applications. Effecting the clarification of fluids is an ancient filtration practice that removes visible particles, variously described as being from 20 or 40 μm in size. Among the most demanding application is the filtrative sterilization of pharmaceutical preparations. This is an important activity whose performance is commonplace and reliable. The segregation of different size particles from mixtures can be managed by using sieves of different mesh sizes (Fig. 1).

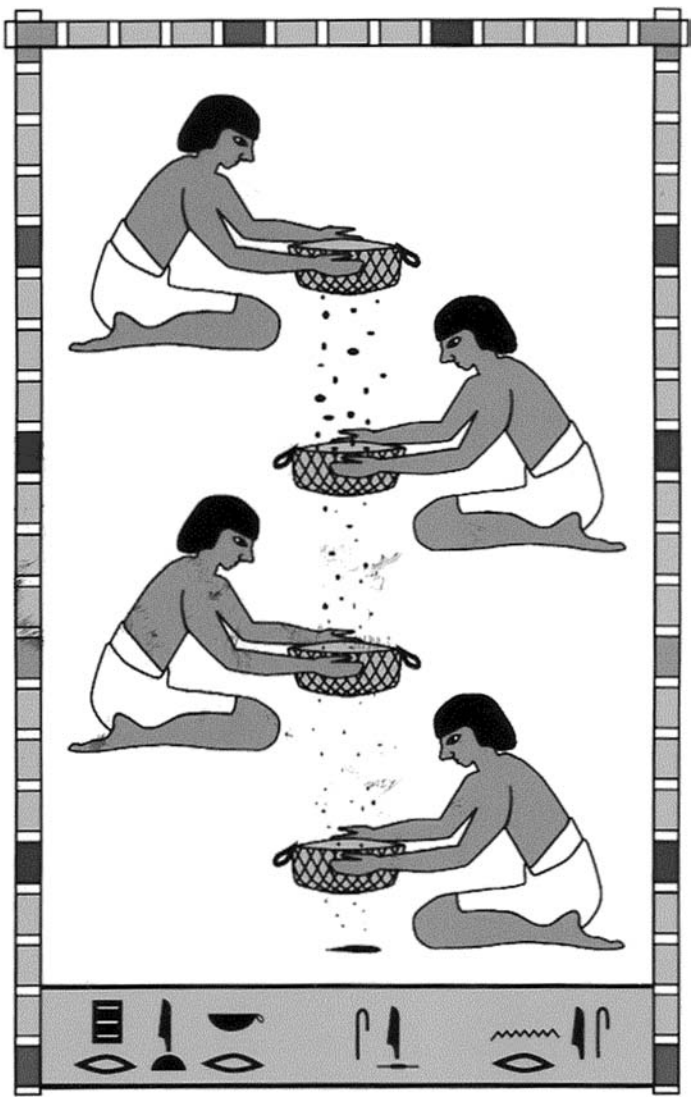


FIGURE 1 *Source*: From Lukaszewicz et al., 1981.

Pore Size Distribution

Earlier there was the belief that size exclusion was the sole mechanism of particle removal by filters. If that were so, namely, if all of a filter's pores were of the same size, the retention of organisms, also essentially of a given but somewhat larger size, would be independent of the challenge density. It was known, however, that retention efficiencies could vary inversely with the organism density of the challenge (Wrasidlo et al., 1983; Wallhäusser, 1979).

Pore size distributions wherein the number of smaller pores outweighs the fewer large pores explains why the efficiency of particle removal in filtrations is dependent upon the challenge density. Only when so great a number of organisms is present as to enable confrontations with the few larger pores, do the organisms escape capture. This despite the fact that hydrodynamic flows do favor the larger pores.

Particles in Dilute Suspensions

Interesting results were forthcoming from filtration studies involving dilute suspensions. Their implications seem important enough to warrant experimental confirmation being undertaken. Organism challenges to membrane filters are generally based on the total colony forming units (CFU) that confront each square centimeter of effective filtration area (EFA). There is reason to believe, however, that the retention results reflect not the total count alone, but also the state of dilution in which the challenge is presented to the filter.

Grant and Zakha (1990) challenged 0.45-μm-rated PVDF membranes with latex particles of different sizes in dilute suspensions. The latex particles averaged 0.605, 0.652, and 0.662 μm in size. The particle concentrations were from 1.2×10^8 to 1.4×10^9 particles per liter. Grant and Zahka associated log reduction values (LRVs) with the concentration of the particles removed by the filters. This was done by periodically measuring the (particle) population in the successive portions of effluent. Grant and Zahka found that the capture of particles initially showed complete retention. This was followed by particle penetration of the filter. This decline in retention, however, gradually slowed to the point where the retention improved with further filtration. These phenomena can be rationalized as follows: Hydrodynamic flow tends to direct the suspended particles to the larger pores; the flow rate being a function of the pore radius to the fourth power. However, larger pores are far less numerous than the smaller retaining pores of the pore-size distribution. On the basis of probability, the particles initially encounter and are retained by the many smaller pores. As these become progressively blocked, small pore availability decreases. The relatively fewer large pores proportionately increase in number. The particles, hydrodynamically directed, begin to find and penetrate them. As filtration continues, pore clogging, and bridging gradually diminish the dimensions of the larger pores. The larger pores having become smaller, the retentivity increases (Fig. 2).

A similar finding was made by Roberts et al. (1990), and Roberts and Velazques (1990) using latex particles (Fig. 3) Emory et al. (1993) confirmed that cross-linked polystyrene latex bead "retention is strongly dependent on particle feed concentration." The course of the curves can be rationalized as follows: The more numerous the particles and the larger their size, the faster is the recovery of the retention from its low point. That is because both size and number tend to more rapidly block the larger pores. When the blockage develops slowly, the observed particle penetration becomes more obvious. A more rapid reduction in the size of the

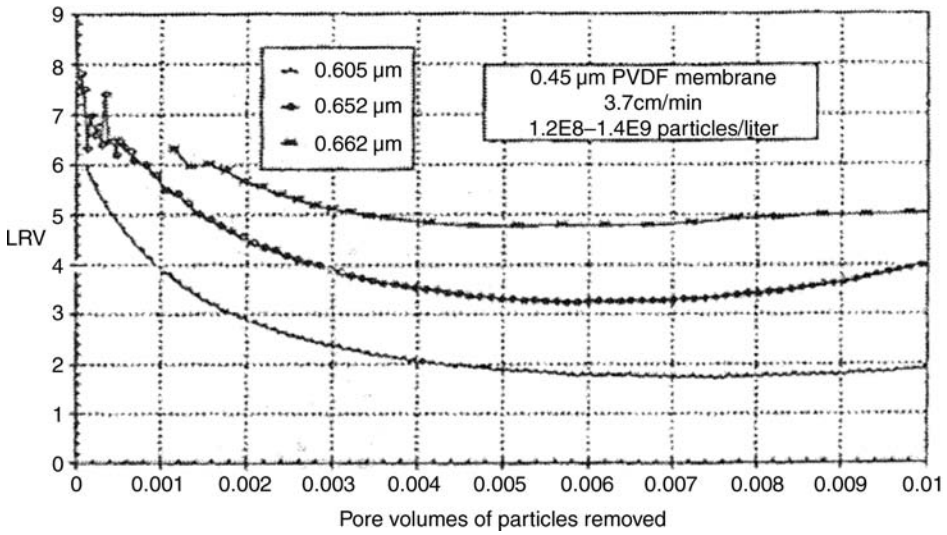


FIGURE 2 Retention of larger particles at high filter loadings. *Source*: From Zhake and Grant, 1991.

larger pores gives the appearance of less discernable penetrations within the time period. It is a matter of the rate at which a sufficient mass of particles reaches the larger pores to block them, and of the number of un-retained particles that are counted in that period of time. So large a number and/or of so large a size can simultaneously arrive at the pores as to give the appearance of immediate blockage. In effect, the period during which the larger pores are open enough to permit particle penetration is so foreshortened as not to allow the penetration of enough particles to be noticed. Thus, the same total number of particles impacting the membrane at different rates can elicit different retentions.

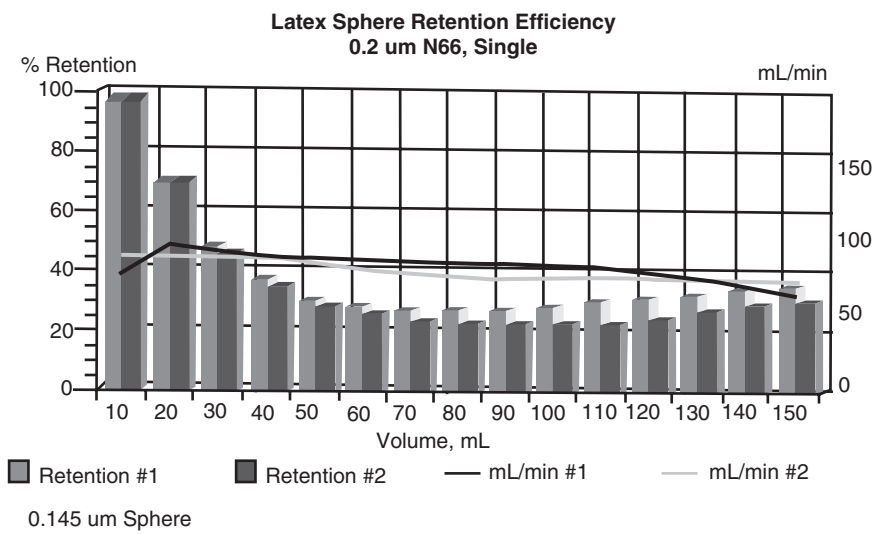


FIGURE 3 Retention of latex particles. *Source*: From Roberts et al., 1990.

The dilution effect not having been investigated to any great extent with organisms, nor even fully with latex particles, remains hypothetical. However, Trotter et al. (2000) in an inquiry regarding the rate at which organism loading affected both the bubble point and the diffusive airflow of a membrane found data supportive of the thesis. Trotter et al. demonstrated the same occurrences attending the filtration of *Brevundimonas diminuta* organisms. Figure 4 is a plot of diffusive airflow versus bacterial loading at 45 psi (3 bar). Initially the airflow rate is constant, but it subsequently decreases gradually as the extent of organism loading increases. Nevertheless, such test behavior depends on the membrane configuration. Double filters are not thus affected because of the upstream filter's acting as a prefilter in reducing the load on the downtream final filter. This decrease in airflow confirms the Grant and Zahka (1990) findings that organisms initially engage the smaller pores because of their greater numbers. The consequent blocking of the smaller pores decreases the filter's porosity relatively little until significant numbers become occluded to air passage. This results in increasing the proportion of larger pores, which in turn, leads to particle passage. Furthermore, the greater the concentration of the organism challenge, the briefer is the interval before the larger pores are obstructed. This equates with the higher latex particle concentrations more speedily causing the clogging that diminishes the diameters of the larger pores; thus, telescoping the time interval for the latex penetration and reducing the possibilities for penetration to occur. This, too, accords with the observations made by Grant and Zahka.

A confirmation of this hypothesis is forthcoming from Duberstein (1979) who reports that "short-term tests with *high concentration challenge levels* (emphasis added) using *P. diminuta* as test organisms" resulted in so large a pressure drop as to effect a blockage of a 0.2 μm-rated membrane without compromise of the effluent's sterility. The blockage occurred at a level of about 10^{13} test organisms per square feet of membrane surface. Wallhäusser (1976) reported that organism breakthrough took place as the total challenge number increased at concentrations of over 10^4/mL (Table 1). Elford (1933) found and confirmed that higher total organism densities resulted in greater probabilities of organism passage (Fig. 5). Thomas et al. (1992) during bacterial challenge experiments with *P. aeruginosa* periodically determined the pore size distribution of the challenged membrane. These investigators computed the numerical integral of the pore distribution function obtained during the challenge interval. A relationship became evident between these values and the volume filtered. From this, the number of organisms challenging the membrane was deduced. Thomas et al. conclude, "Assuming only the sieve retention

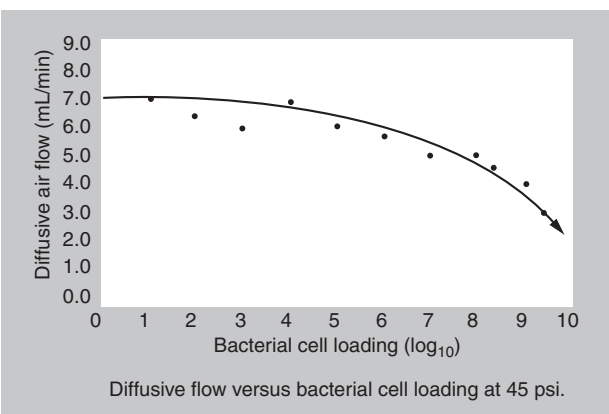


FIGURE 4 Plot of diffusive airflow versus bacterial loading at 45 psi. *Source*: From Trotter et al., 2000.

TABLE 1 Dependence of Organisms Breakthrough on Initial Organism Concentration

Initial *P. diminuta* conc.	10^3/mL		10^4/mL		10^5/mL	
Filtrate (ml)	0.2-μm rated	0.45-μm rated	0.2-μm rated	0.45-μm rated	0.2-μm rated	0.45-μm rated
100	0	0	0	1	1	1000
200	-	-	2	4	4	
1000	0	0	9	25	17	(10^4)
Filtration time for 1000 ml	6' 52"	2' 27"	2' 12"	2' 30"	3' 15"	8'

Source: From Wallhäusser, 1976.

mechanism is at work, the membrane's pore-size distribution then assumes a higher degree of importance, because it provides a measure of the probability that such bacteria will encounter pores large enough to allow their passage." The influence of the challenge dilution, previously demonstrated for latex spheres and silica particles, is now shown as conceivably applying to organisms as well.

Dilute Challenge Implications

There are at least two conclusions to be drawn from the dilute challenge effect. First, the EMEA requirement that final filters not be confronted with more than 10 CFU per ml could be a greater test of the retention capabilities of a filter than the FDA's 1×10^7 CFU

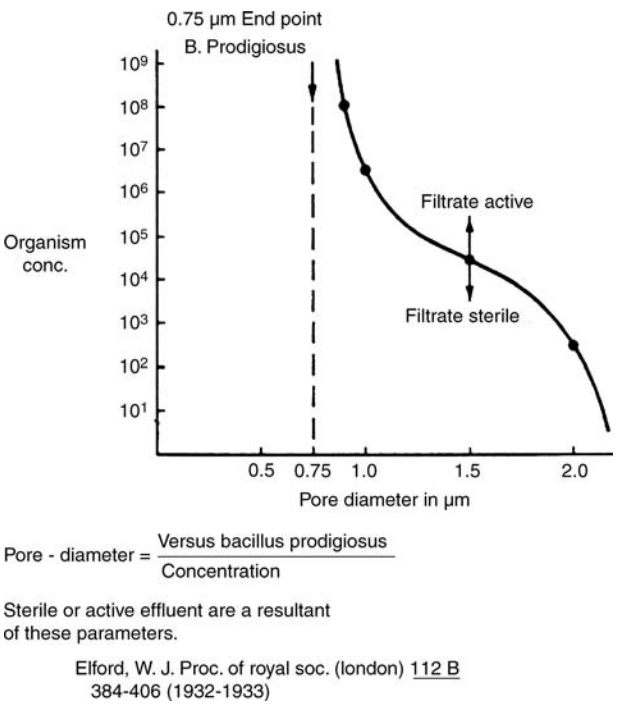


FIGURE 5 Higher total organism densities resulting in greater probabilities of organism passage. *Source*: From Elford, 1933.

per cm^2 EFA which is usually performed with only some 2L of water. With reference to the choice of 0.1- or 0.2-/0.22-μm-rated membranes as sterilizing filters, it was stated that the unnecessary use of the tighter filter would result in decreased flow rates and their accompaniments of possible premature blockage, etc. However, the pore and organism size relationship that underlies making an intelligent selection posits knowing what the organism size will be in its suspending liquid, given that size shrinkage may occur. This would require a pre-filtration sizing of the organism(s) of interest after exposing them to the liquid vehicle for a duration at least equal to that required for the processing step itself. This, in turn, would necessitate a far more diligent bioburden assessment than is customarily performed. In short, an educated choice of the filter would rely upon a validation performance.

The Largest Pores

Mostly, the pore size distribution remains a subsidiary concern despite its influence on the retention picture (Zahke and Grant, 1991; Jornitz and Meltzer, 2001. This is so because the focus is on particle passage which is seen as occurring through the largest pores regardless of the overall distribution. Hence, the emphasis on the bubble point measurement of the set of largest pores. There is reason to believe that, despite their relative paucity, the larger pores are early on engaged because of the preferential flow through larger orifices (Jornitz and Meltzer, 2004; Mouwen and Meltzer, 1993; Grant and Zahke, 1990). This is not necessarily so, but when it is organism passage may occur. In this view, the measurement of the smaller pores, those adequate for the sieve retention of the organisms, can safely be ignored. One factor in particular had delayed acceptance of the dependence of organism retention on the challenge density as being due to pore size distribution.

Early-on in membrane usage it was held that the pore size distribution was of negligible importance. The pore size distribution had been explored by mercury porosimetry and had been reported to be a narrow ± 5%. The 0.45-m-rated membrane was said to be ± 0.02 μm in its distribution. "It reflects an extraordinary degree of uniformity" (Dwyer, 1966). Subsequently, Badenhop et al. (1970) and Marshall and Meltzer (1976) determined that the casting process of membrane manufacture produces filters having pores with a relatively narrow pore size distribution that, in the few cases investigated, are essentially Gaussian in shape (Fig. 6). Marshall and Meltzer's measurements showed that the largest pore was about double the size of the mean flow-pore value for the membranes examined.

As said, the widespread early-on ignoring of pore size distributions helped promote the belief, for all practical purposes, in the exclusivity of sieve retentions. The pore size distributions among 0.2-/0.22-μm-rated membranes prepared of different polymers by seven different filter manufacturers is illustrated in Figure 7 (Meltzer and Lindenblatt 2002). Latex beads were employed by Wrasidlo et al. (1983) in retention studies to obtain the flow pore size distributions of membranes (Table 2).

Adsorptive Effects

Although adsorptive sequestration was first seriously proposed as an important mechanism of organism retention only in 1979 by Meltzer and his associates, it had previously long been recognized that filters of many chemical compositions, including polymeric membranes, are capable of adsorbing various molecular entities. As far back

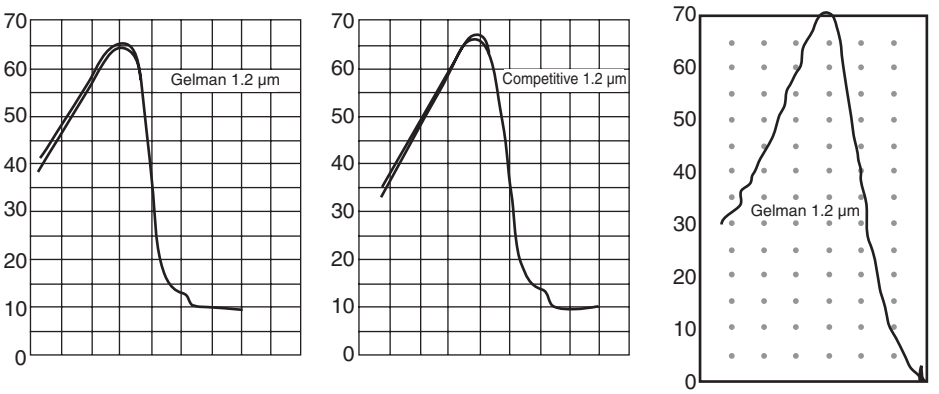


FIGURE 6 Automated flow pore measurement comparison of Gelman and competitive 1.2 µm membranes. *Source*: From Lukaszewicz, et al., 1981.

as 1909, Zsygmondy pointed out that the filter surface has a certain adsorbing capacity that must be satisfied before unhindered passage of the dispersed phase through the filter occurs. Numerous investigators have since noted many specific adsorptions. In 1927, Kramer worked with "bacterial filters" composed of derivatives of silicic acid, namely, sand, porcelain, and diatomaceous earth. Such Berkefeld siliceous filters are definitely negatively charged. He also used plaster of Paris filters composed of calcium carbonate and magnesium oxide. Such filters are positively charged, He found that the filters passed same-charged entities, but retained those of opposite charge. Thus, Congo Red dye, negatively charged, is retained by the plus charged plaster of Paris filters, but passes through the siliceous filters of minus charges. Congo Red upon slight acidification is altered to its blue colored manifestation that is positively charged. As such, it is retained by the negatively charged Berkefeld filters, but passed through the plus charged MgO and $CaCO_3$ filters. The charge relationships in filtrations are evident. Kramer worked with viruses and bacterial toxins, not with bacteria. He makes clear, nonetheless, that he believes the charge neutralization effect he demonstrated would apply also to bacteria.

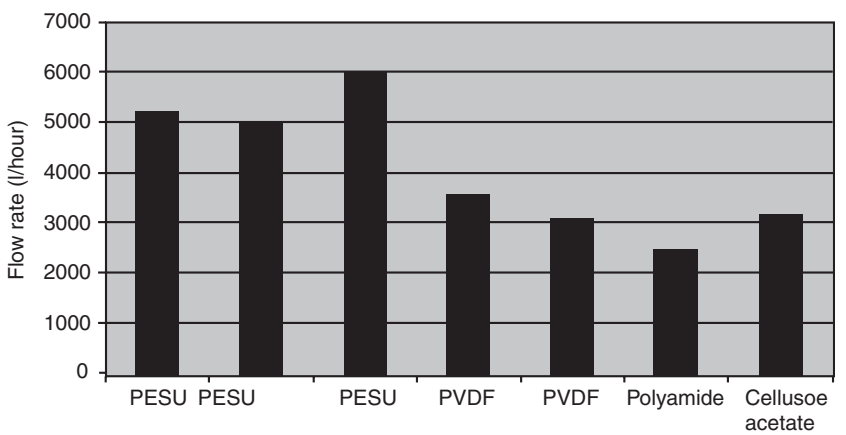


FIGURE 7 Pore size distributions among 0.2/10.22-µm-rated membranes prepared of different polymers. *Source*: Jornitz, 2005.

TABLE 2 Retention of Various Size Latex Particles for 0.2 μm-Rated Membranes

Latex particle size (μm)	0.091	0.198	0.305	0.460
Membrane type	Percent retention			
Asymmetric polysulfone	54.3	100	100	100
Charge-modified nylon	10.5	100	100	100
Polycarbonate (track-etched)	6.3	100	100	100
Polyvinylidene difluoride	23.4	19.2	84.5	100
Cellulose esters	17.7	25.1	48.6	100
Nylon 66	1.0	1.0	1.0	100

All solutions 0.04% latex in 0.05% Triton X-100.
Source: From Wrasidlo and Mysels, 1984.

The mechanism of the adsorptive retention is the attractive forces of opposite charges. Kramer explains the mechanism as being due to the Helmholtz double layer. Also known as the electric double layer, it is discussed below.

Elford (1933) reported that dyes could adsorptively be removed from true solutions by collodion membranes; cellulose nitrate being a most adsorptive material. The strong adsorption tendencies of the cellulose nitrate polymer had also been noted by Elford (1931) in the case of viruses. The use of membrane filters to adsorptively collect and isolate nucleic acids, enzymes, single-strand DNA, ribosomes, and proteinaceous materials in scintillation counting operations is well established. Moreover, such adsorptive retentivity is utilized nowadays by introducing chromatography and membrane adsorber steps into the downstream purification stages of fermentation processes. Bovine serum, antigen/antibody, and antibody complex, and specific binding and receptor protein adsorption to cellulose nitrate has been shown to occur. Berg et al. (1965) investigated the adsorption of both inorganic and organic compounds upon polymerics such as cellulosic filter papers, nylon, polyethylene, and cellulose diacetate dialysis membranes.

That water-soluble organics could adsorptively be removed from aqueous solutions by filters was observed by Chiou and Smith (1970). These investigators were thus led into a rather thorough study of such adsorptions by filters. Udani (1978) and Brose et al. (1994) studied the adsorptive sequestration of such preservatives as benzalkonium chloride, chlorocresol, and chlorhexidine acetate from their solutions by membrane filters. The adsorptive removal of flu vaccine impurities and antibodies onto membrane filters has been reported (Tanny and Meltzer, 1978). Inorganic particulate matter can be removed filtratively through the adsorption mechanism. It is thus well documented that molecules and materials can be adsorbed onto filters, to become filtratively removed thereby.

Adsorption of Organisms

The adsorptive bonding of numerous entities, including organisms, to solid surfaces is noted in the literature. Some 80 years ago Kramer (1927) indicated that size exclusion was not the exclusive mechanism whereby membranes retained organisms. Four decades ago Nash et al. (1967) stated, “These filters do not act as mechanical sieves alone, since the electrical charge on the particle (bacterium, virus, etc.) and the composition of both filter and the suspending medium will play a part in determining

filterability. The ability to pass through filters is related to particle size in only a crude way." Pertsovskaya and Zvyagintsev (1971) found that different groups of different bacteria are adsorbed by polymeric films composed of polyamides, polyacrylates, polyethylenes, or cellulose acetate. That various bacteria adsorb onto various surfaces was also disclosed by Gerson and Zajic (1978). Hjertin et al. (1974) studied the adsorption of yeasts on nonionogenic, hydrophobic agarose, and the column adsorption of *S. typhimurium*.

Zierdt and his associates in 1977 at the National Institutes of Health noted that both Gram-negative and Gram-positive organisms were retained on the surfaces of polycarbonate, and cellulose acetate membrane filters of pore sizes much larger than the bacteria. The organisms involved in the studies were *Escherichia coli* and *Staphylococcus aureus*. The adsorptive bonding of the bacteria to the polymeric filter surface withstood the mechanical and desorptive actions of washings with buffer solutions (Zierdt et al., 1977). SEM photographic evidence is shown in (Fig. 8) of 0.8 μm *S. aureus* organisms retained on the horizontal surface of the membrane, and upon the vertical lips of its pores. The membrane was a (track-etched) polycarbonate of 12-μm-rated pore size. Zierdt et al. (1977) found that a higher percentage of organism retentions occurred at challenge levels as low as 500 CFU to 1,000 CFU/ml than took place at the higher levels of 10^8 to 10^9 CFU/ml. At the higher densities increasing number of *Escherichia. coli* passed through the filter, although more were retained. Again, these findings accord with adsorptive sequestration effects, not with sieve retentions. Leahy and Sullivan's (1978) SEM shows *B. diminuta* pendant from glass fibers in circumstances unattributable to sieve retention (Fig. 9). The SEM photographs of organisms retained by filters despite the absence of sieving conditions confirms that other capture mechanisms are operative. Tanny et al. (1979) demonstrated that the ability of 0.45- m-rated membranes to contain challenge densities of 2×10^7 CFU/cm^2 of filter area depended upon the pressure differential being reduced to 0.5 psi (0.3 bar) (Table 3). Sterile effluent was not obtained at the higher delta pressures of 10 and 15 psi. This dependence of organism capture upon the transmembrane pressure accords with adsorptive sequestration effects, but not with sieve retentions. These investigators, therefore, challenged the exclusivity of sieve retention as the mechanism of organism removal. They postulated that the retention of *B. diminuta* by 0.45-μm-rated cellulose acetate membranes involved adsorptive sequestration.

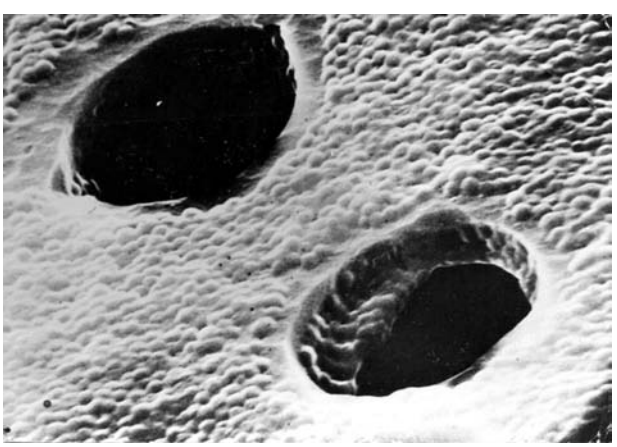


FIGURE 8 *S. aureus* retained on surfaces of polycarbonate membrane. *Source*: From Lukaszewicz et al., 1981; Zierdt et al., 1977.

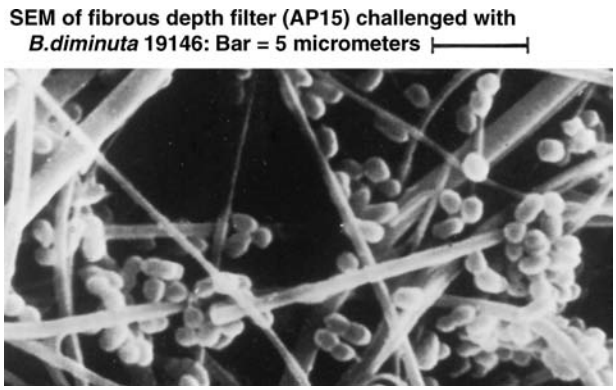


FIGURE 9 *B. diminuta* pendant from glass fibers. *Source*: From Lukaszewicz et al., 1981; Leahy and Sullivan, 1978.

Some Operational Influences

Differential Pressure

To the extent that particle removal is dependent upon sieve retention, the filter efficiency, in terms of the percentage of the total particles that are removed, should not be affected by the differential pressure. This is because sieving is essentially independent of the challenge level, or of the flow rates as dictated by the differential pressure. There are some negative effects, however. Compactions caused by higher differential pressures may render filter cakes less penetrable by the fluid. Foreshortened throughputs may result. Slower rates of flow may also result from the densification of the diffused polarized particle layer suspended in front of the filter (Fig. 10). Where particles smaller than the pores are present, filter cake densification, as also cake buildup, should progressively increase the filter efficiency by retaining smaller particles. Differential pressure can have a profound effect upon filter efficiency where particles are subject to adsorptive removals. Increased liquid flow rates, the product of higher ΔPs, reduce the residence time of the particle in the pore passageway. This diminishes the prospects for its adsorptive sequestration to take place. The longer the mutual exposure of particles and pore-wall surfaces, the greater the chances

TABLE 3 Pressure Dependent Retention Performance

Operating pressure (psi)	Total filteration time for 2000 mL min: sec	500 mL	1000 mL	1500 mL (org. 100/mL)	2000 mL	Avg. no. of org. in filtrate/mL
5	189:30	0	0	0	0	
5	75:00	4	12	7200	7200	0
5	304:00	0	0	0	0	
15	108:27	0	13	19	39	
15	69:30	3	2	0	7200	10-20
15	43:58	6	15	12	11	
30	18:35	93	91	61	66	
30	16:12	38	34	39	52	50-100
30	50:02	7200	7200	7200	7200	

Cellulose triacetate 0.45μm-rated membrane challenged with *B. diminuta* suspension of 10^5 org/cm 2000 mL over 9.6 cm^2 available surface (47 mm disc). Total organism challenge level 2×10^7 org/cm^2.
Source: From Tanny et al., 1979.

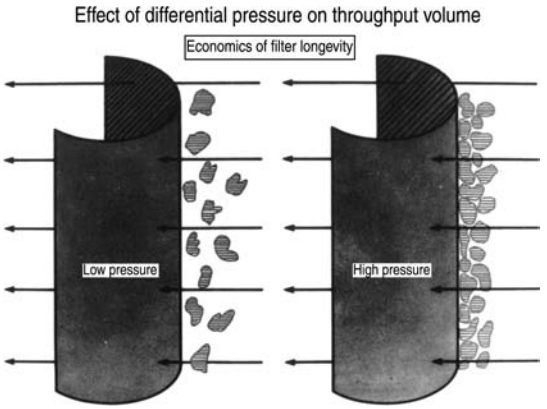


FIGURE 10 Effect of differential pressure on throughput volume. *Source*: Courtesy of Capitola Presentations.

of their adsorptive connection. Lower delta pressures increase retentions where the adsorption mechanism is involved because longer residence times increase the probabilities of pore wall encounter, and of resulting particle captures. This accords with the experience that employing lower ΔPs tends to increase filter efficiencies.

The overall effect of higher Δ*P*s on filter efficiencies will vary depending upon the extent of particle loading, and the proportion of smaller and larger particles relative to the filter's pore size distribution. The permeability of a filter cake depends also upon the packing pattern of the retained particles. This, in turn, reflects the numbers, sizes, and shapes of the particles involved (Meltzer, 1987; Wrasidlo and Mysels, 1984). This touches upon the choosing of prefilters, a subject not of this writing.

Temperature

Temperature has several effects that require consideration. Temperature gives a greater amplitude to particle diffusion, promoting the likelihood of adsorptive pore-wall encounters. On the other hand, at a given differential pressure a fluid will flow faster at more elevated temperatures, thereby reducing the residence time within the filter and thus working against adsorption. By reducing the duration of the filtration, higher temperatures become the equivalent of higher differential pressures. Nevertheless, overall, adsorption from aqueous solutions seems generally favored by higher temperature. It results in a more rapid rate of adsorption to a lower degree or capacity.

Temperature has been shown to enhance the efficiency with which the smaller particles of an AC fine test-dust suspension in water are removed by membrane filters. This was experimentally determined in a study wherein, to eliminate the effect of viscosity, polypropylene glycol was added to the aqueous solution to keep its viscosity constant even as its temperature was raised (Johnston, 1985). The increase in the efficiency of small-particle captures caused by temperature is ascribed to the increased amplitude of the diffusion of these particles as caused by their higher thermal energies. Increased pore–wall encounters and concomitant adsorptive sequestrations result.

If the sizes of pores and organisms are presumed to be unaffected by temperature, the sieve removal of organisms should not be affected. if so, it would seem, therefore, that it is the adsorptive effects that must be reduced by temperature elevations. The rationalization has the higher temperature reducing the viscosity that in turn, reduces the particle's resident time within the pore's passageway, which reduces the amount of retention. Higher temperature in this regard has the same effect as increasing the differential pressure.

Viscosity

Viscosity finds expression in slower rates of flow. This will prolong the residence time of particles within pores. This should incline towards enhanced adsorptions. However, the viscosity will also reduce the likelihood of pore wall encounter by limiting the rate of particle travel imparted by the collisions produced by Brownian motion. Inertial impactions will be similarly affected. Viscosity is amenable to moderation by increase in temperature.

The viscosity of a pharmaceutical preparation may be so sufficiently high as to make impractical its flow rate through the 0.2-/0.22-μm-rated membranes usually employed in filtration sterilizations. In the event, repetitive 0.45-μm-rated are often used. This practice could compromise the sieve retentions of given organisms, and may impose stronger reliance upon adsorptive arrests. Such organism removals will be favored by the slower flows of the viscous material.

Water Solubility

Hydrophilic and Hydrophobic

The terms "hydrophilic" and "hydrophobic," respectively, from the Greek, denote a fondness or love of water, and an antipathy to water. Molecules exhibit these qualities according to the polarity or non-polarity of their constituting atomic arrangements. Generally, polarity derives from oxygen atoms such as are present in ethers, esters, alcohols, carboxylic acids, etc. The oxygen atom is strongly electronegative. In its participation in covalent bonding it retains more than its share of the bonding electrons. The polar areas result from the partial charges caused by such unequal sharing of bonding electrons. The partial electrical charges (discussed in the following sections) result in hydrations, in aqueous solubility, and in other manifestations of hydrogen bonding. The non-polar molecules, lacking obvious electrical charges, have no affinity for H-bonding, or, presumably, for the mutual interactions of opposite electrical charges. However, complex structures such as proteins, polymers, and the organism and filter surfaces composed of them may have multiple sites of polar and non-polar character.

It is possible to generalize regarding the adsorption of materials from aqueous media by viewing the adsorptive phenomenon as being in competition with the tendency of the material to remain in solution. Water solubility derives from the polarity, the partial electric charges, of molecules that enable their adsorptive interactions with oppositely charged sites on water molecules. The hydrogen bond, soon to be explained, is the agency of this interaction. The less attraction its molecules have for water molecules, the less water-soluble is a substance and the easier it is to remove it from solution by adsorption. By this measure, less ionized or less-polar molecules are easier to adsorb from aqueous solutions. They have fewer or no alliances with water molecules to hold them back from the attractions of stronger adsorptive sites such as may exist on filter surfaces.

Wetting Action of Surfactants

The hydrogen bond is the source of water-wetting. It figures in the action of wetting agents. These are molecular structures which are polar at one end or site and non-polar at another. The molecule of a classic type wetting agent or surfactant consists of a very hydrophobic alkylated aromatic moiety, such as dodecylbenzene, attached to a highly hydrophilic portion composed of repetitious units of ethylene oxide or propylene oxide. The non-polar hydrophobic end of the surfactant molecule is hydrophobically adsorbed onto the hydrophobic surface of the suspended particle whose water-wetting is ultimately desired.

The hydrophilic portion of the surfactant molecule extends into the aqueous solution where its repetitively spaced etheryl oxygens, (–O–), the seat of its polarity, feature the electronegativity of their unshared electrons that form hydrogen bonds with the water molecules.

The surfactant molecules mediate between the particle's hydrophobic, non-polar surface and the water, the hydrophilic medium. In effect, the arrangement is a water-wettable surface adsorptively grafted onto the hydrophobic particle which, as a result, is now amenable to the aqueous medium.

Surfactant Particle Size Enlargement

The findings of Bowman et al. (1967) wherein penicillinase occupied the adsorptive sites of a filter that would otherwise have served to retain *B. diminuta* have been discussed. Surfactants can likewise preempt the adsorptive sites of filters to deny latex particles their access. In studies involving the retention of latex particles serving as surrogates for organisms, it was found that retention efficiencies decreased in the presence of surfactants. An alternate explanation is possible, namely, that surfactant deposited on the latex particles increases the energy barrier to their coming together. The resulting steric stabilization, also called entropic stabilization, enlarges the distance of their separation to such an extent that the weak forces of attraction cannot overcome it.

Pall et al. (1980), in reporting that the presence of surfactant diminished the latex bead retention, noted that different surfactants did so to different extents. Emory et al. (1993) corroborated that not all surfactants have the same effect on a given membrane. Confirmation of Pall et al.'s findings were made by Wrasidlo et al. (1983), in respect to both pH, and surfactant (Tables 4 and 5). Tolliver and Schroeder (1983) compared the retention of 0.198 μm latex beads suspended in water, with those suspended in an aqueous solution of 0.05% Triton X-100. The comparisons were made using various commercially available 0.2-μm-rated membranes. Table 6 shows differences in results between the two vehicles. The dissimilarity is greatest for the nylon 66 membrane. The polyamide polymers are known to exhibit adsorptive interactions with surfactants that interfere with, for example, the uptake of proteins. The action of surfactant in differentiating among the extents of latex particle retentions in otherwise similar situations is taken as a confirmation of the adsorptive sequestration mechanism.

Significance of Mechanism

Size exclusion is so dominantly the mechanism of particle retention that it is erroneously still regarded by some to be the exclusive mode of organism (particle) retentions. This is

TABLE 4 0.198-μm Latex Percent Retention for Various 0.2 μm-Rated Membranes as a Function of pH

Filter type	Bubble point	pH 4	pH 6	pH 8	pH 9
Asymmetric polysulfone	51	100	100	100	100
Polycarbonate (track-etched)	63	100	100	100	100
Polyvinylidene difluoride	55	86.8	74.8	79.5	67.3
Cellulose esters	58	36.3	89.4	23.0	31.3
Nylon 66	45	99.9	82.1	23.7	28.4

Source: From Wrasidlo et al., 1984.

TABLE 5 Retention (%) of 0.198 μm-Spheres by Various 0.2 μm-Rated Membrane

Filter type	In water	In 0.05% Triton X-100
Polycarbonate	100.0	100.0
Asymmetric polysulfone	100.0	100.0
Polyvinylidene fluoride	74.8	19.2
Nylon 66	82.1	1.0
Cellulose esters	89.4	25.1

Source: From Toliver and Schroeder, 1983.

the situation despite that some 70 years ago Elford (1933) wrote, "the importance of adsorption in filtration has long been recognized." Nevertheless, the presumed absolute certainties of sieve retention retain their blandishments. There are advantages to the sieve retention mode of particle arrests. Where the mechanism of particle retention can be selected, sieve retention or size exclusion should be the choice. Its certainty is less conditional than the alternative mechanisms. Its restraint on the passage of an organism through a pore depends essentially only on their size relationship. Indeed, the selection of a filter for an application is commonly made with the aim that its pore size rating will be suited to the sieve removal of the suspended particles.

Sieving is free of the many influences that govern adsorptive sequestrations, such as the number of pores, the challenge density, the adsorptive propensity of the polymeric filter, the differential pressure, temperature, viscosity, ionic strength of the solution. The axiomatic nature of the size exclusion mechanism is assuring in its simplicity. Thus, when non-sterile effluent results from the use of 0.2-/0.22-μm-rated membranes (Sundaram et al., 2001, Part I), the advocacy is made to use membranes of lower pore size ratings, more assertive of size exclusions. The 0.1-μm-ratings are championed as alternatives to the more conventional use of the 0.2/0.22 μm variety despite that the organisms escaping capture by the latter are not necessarily retained by the former. There are applications that do require the use of tighter filters. However, a needless penalty in flows is incurred when the proposed substitution is gratuitous (Kawamura et al., 2000).

The use of membranes that are tighter than needed to perform desired organism removals unnecessarily invite reduced throughputs; as also premature filter blockage resulting from retained particles whose removal is not considered necessary for the drug's purity or efficacy. The rate of flow is ineluctably reduced. Its restoration to practical levels may require longer processing times, higher applied pressures, or more extensive filter areas. As a guiding rule, a membrane of as large a pore size rating as will assure the desired extent of particle retention should be used. The flow that follows this choice is accepted as an inherent consequence.

TABLE 6 Impact of Pressure on Passage (β Ratio)

		β Ratio		
Filter type	Pore size (μm)	0.5 psid	5 psid	50 psid
GS	0.22	$>10^{10}$	$>10^{10}$	$>10^{10}$
HA	0.45	10^8	10^7	10^6
DA	0.65	10^4	10^4	10^3
AA	0.80	10^2	10^1	10^0

Source: From Leahy and Sullivan, 1978.

Absoluteness in the sense that employing a given filter, of whatever pore size rating, will invariably yield sterile effluent is probably unattainable. The ultimate filtration results depend upon the specifics of the membrane, of the organism type, of the fluid's composition, and of their interactions, plus the choice of the filtration conditions.

It is not necessary to understand the adsorption effects; provided that the omission does not equate with an ignorance of their influences. If one understood only the operation of sieve retention, then it would be possible to conclude that the challenge density is not an important factor in filtrations (especially when pore size distribution is seldom of concern.) It may even be concluded that differential pressure is not a prime determinant of retention (unless its level is high enough to distort the particle, allowing for its permeation of the filter). The efficacy of low differential pressure applications in achieving enhanced retentions is well understood by filter practitioners; but perhaps largely as a rule of thumb. As such it has value. However, comprehending that the differential pressure governs the residence time of the microbe within the pore pathway, that this in turn reflects on the probability of pore wall encounters, and that this can influence the likelihood of adsorptive captures, offers the advantages that derive from understanding the phenomena involved.

It might not have mattered if Bowman and associates could have filtered *B. diminuta* from a penicillinase solution without understanding the retention mechanism. It turned out to be important, however, to learn that proteinaceous materials, by way of a different mechanism, could interfere with the retention of *B. diminuta*, and to understand how by use of a second mechanism this interference could be avoided.

Achieving maximization of adsorptive retentions is conditional, as stated, upon attaining certain stipulations, namely, the use of membranes of suitable polymeric compositions, amenable organism types, the imperviousness of both organisms and pores to size alterations by the fluid, and securing the proper filtration conditions. Not enough is yet known about the interactions and relative importance of these factors to permit their optimization. By contrast, the certainty of sieve retention seems utterly simple. However, it too has its unexplored dimensions, for example, pore size distributions, particle-concentration effects, particle deformations under pressure, rates of organism size alterations, etc.

The adsorptive sequestration mechanism is not compromised by the complexity of its background. Where particle retention by either mechanism takes place, it remains viable and dependable given the fulfillment of the necessary operational conditions. In any case, the attainment of organism retentions by whatever mechanism requires validation, confirmation forthcoming from documented experimental evidence. As regards the filtrative removal of organisms by adsorptive sequestration, a fuller substantiation of the mechanism will be detailed below.

SUPPORTIVE EXPERIMENTAL FINDINGS

The dependence of adsorptive sequestration on the differential pressure is illustrated in Table 3 and Figure 11. In the latter illustration, an organism small enough to enter the membrane pore can meet one of two fates; it can either emerge with the convective stream, or, because of Brownian motion, it can contact the pore wall to become adsorptively attached. The longer its residence time within the pore, the greater the probability of its pore wall encounter, and adsorptive interaction. Thus, differential pressure is a process condition that influences a filter system's retention qualities. The lower the stream velocity, as governed by the differential pressure, the longer the residence time. The viscosity of a

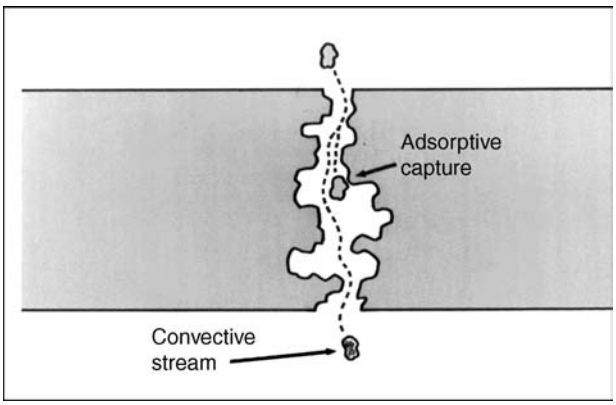


FIGURE 11 Alternate paths for particles entering pores. *Source*: Courtesy of Capitola Presentations.

fluid in its capacity to attenuate the mean free-path of Brownian motion is also a property that influences adsorptive captures. So, too, is temperature, in that it is a moderator of viscosity.

The retention of particles of different sizes and shapes may be affected differently, and to different extents by these fluid properties. Sieving, the size-discriminating mechanism, is independent of the challenge level. Its only requirement is that all the particles be larger than the largest pores. However, the adsorptive particle arrests depend upon a conjunction of the several conditions that define a filtration, including the adsorptive bonding of the particle and membrane surfaces. Lacking the certainty of a conjoining of the two surfaces introduces a probability factor into the adsorptive sequestration operation. Its results do depend upon the challenge density. The larger the number of organisms that essay passage of the filter, the more will emerge with the effluent. Thus, the higher challenge densities do more severely test the filter.

Elford's Findings

Elford (1933) confronted filters of different pore size ratings with organism challenges of different severities. He observed, as depicted in Figure 5, that below a certain pore diameter the filter completely retain as many as 10^9 *Bacillus prodigiosus* (now called *Serratia marcescens)*. Only at pore diameters larger that necessary for sieve retention is the efficiency of filtration dependent upon the organism challenge level. Above Elford's "end-point" or critical pore size, adsorptive sequestration becomes the capture mechanism, reinforcing the effects of sieve retention, but subject to the number of the particles that are present. The filter efficiency is greatest where the challenge level is lowest. Particle capture is then a matter of probability; the larger the number of particles, the more likely that some will escape capture. For this reason, too, final filters can be regarded as polishing filters; cleaning fluids already cleaned by prefilters.

Investigators have noted the dependence of retention upon the particle density. Such ought not be the case with sieve retentions where the organism/pore size relationship is the determining factor unless a pore size distribution is involved. The rationalization is that the organisms must be so numerous as to ensure the probability of an encounter with the occasional large pore. Likewise, the dependence of the extent of

retention upon the differential pressure probably bespeaks an adsorptive arrest. It could occur also in a sieving context if the organisms were distorted and forced through the filter pores by the pressure differential, although this is an unlikely event (see section "Wallhäusser's Findings").

Bowman et al.'s Findings

An early indication that sieve retention was not the universal means of filtratively removing organisms resulted from investigations by Bowman et al. (1967). It was found that 0.45-μm-rated membranes composed of the mixed esters of cellulose, then considered the "sterilizing" filters, retained *B. diminuta* except when penicillinase was present in the preparation. It is difficult to account for the action of the penicillinace in terms meaningful to the sieving phenomenon. In the presence of the protein it required the then newly devised 0.22-μm-rated membrane to sterilize the preparation. It was rationalized that the tighter filter affected retention by the sieving mechanism, whereas the more open membrane retained more so by adsorption, provided the adsorptive sites were not competitively occupied by the protein molecules. This experience provided an early recognition of the adsorptive sequestration mechanism's relevance to pharmaceutical filtrations. It also marked the origin of the 0.22-μm-rated membrane as the "sterilizing filter," and the acceptance of *B. diminuta* as the model for small organisms likely to be present in pharmaceutical bioburdens.

Wallhäusser's Findings

Shown in Table 1 are Wallhäusser's findings (1976, 1979) that confirm that organism retentions can reflect the inverse of their numbers. At the time of this experimental investigation the exclusivity of sieve retention and the absoluteness of membrane filtration was in vogue. Wallhäusser's work proved the actual situation to be otherwise. The compromising of sieve retention can be explained on the basis of pore size distribution. Enough organisms need be present to encounter the fewer larger pores in order to avoid capture.

Leahy and Sullivan's Findings

The work of Leahy and Sullivan (1978) provides a concise relationship among pore-size ratings, applied differential pressures, and organism challenge levels for mixed esters of cellulose membranes. As shown in Table 7, mixed esters of cellulose membranes of 0.22-μm-rating exhibit LRVs of 10 against *B. diminuta* challenges whether at applied differential pressures of 0.5 or 50 psi (0.33 or 3.3 bar). That the capture mode for the 0.22 (0.2)-μm-rated membrane is sieve retention is attested to by its freedom from the pressure differential influence. Interestingly, Aicholtz et al. (1987) demonstrated the complete retention of *B. diminuta* ATCC-19146 by 0.22 (0.2)-μm-rated membrane, even at 55 psid (3.7 bar), confirming sieve retentions by the less open membranes.

However, Leahy and Sullivan found that the same type of filter in its 0.45-μm-rated manifestation shows a LRV of 8 at 0.5 psid, a LRV of 7 at 5 psid, and a LRV of 6 at 50 psid. The 0.65-μm-rated membrane, and its 0.8-μm-rated counterpart show the progressively increasing influence of the applied differential pressure level on the organism retention. This is a manifestation of adsorptive sequestration.

TABLE 7 Flu Vaccine Filtration Volume in Mls/seconds

0.45 μm Mixed cellulose esters			Mixed cellulose esters		
Manuf. I			Manuf. II		
36/90	38/90	33/90	28/90	25/90	30/90
38/120	41/120	34/120	31/120	27/120	34/120
(Titers 64, 65%)			(Titers 64, 65%)		
0.45 μm Cellulose triacetate			0.45 μm Dynel-type		
40/90	46/90	40/90	64/90	48/90	52/90
42/120	50/120	42/120	70/120	53/120	57/120
45/180	55/180	43/150	78/180	57/180	63/180
	58/210		80/210		
(Titers 90, 91, 91%)			(Titers 89, 87%)		

Source: From Tanny and Meltzer, 1978.

Leahy and Sullivan (1978) confronted the 0.45-μm-rated membrane with 10^{10} *B. diminuta*, some 10 million organisms. At 0.5 psi (0.13 bar), only about 100, at most, failed to be retained. At 50 psi (2.3 bar), some 10,000 escaped arrest. If one assumes that the difference in the numbers retained at 0.5 and 50 psi may be considered those captured due to adsorption, then even for the 0.45-μm-rated filter, retention of *B. diminuta* is essentially due to sieving. As Carter and Levy (1998) point out, microbial retention efficiency is directly proportion to bubble point values, indicating the prevalence of sieve retention as the capture mechanism. Adsorptive sequestration serves as a reinforcing mechanism, making more certain the organism removal by the filter.

Tanny et al.'s Findings

The contribution of Tanny et al. (1979) was the illustration that 0.45-μm-rated cellulose acetate membranes sustained challenges of 2×10^7 CFU/cm^2 EFA at a pressure differential of 0.5 psi (0.3 bar) but not at higher delta pressures (Table 3). This dependence of organism capture upon the transmembrane pressure accords with adsorptive sequestration, but not with sieve retentions. This finding challenged the then credited belief in the exclusivity of sieve retention as the mechanism of organism removal. Tanny et al. postulated that the retention of *B. diminuta* by 0.45-μm-rated cellulose acetate membranes involved adsorptive sequestration.

Definition of "Mechanism of Retention"

Present understanding of the particle retention mechanisms is strongly based on the sieving effect that results from size exclusions wherein the particle's larger size makes impossible its passage through the pore. There is also the recognition that adsorptive influences, electrical in nature, play a role in the retention of smaller particles. Some consider a mechanism to be the manner in which the particle and filter surfaces come into contact with one another. Thus, there is the "gravitational mechanism" of particle capture; so called because it explains the settling of a particle onto a *horizontal* filter surface. It is not necessary to postulate a bonding force between the two surfaces other than that exercised by gravity itself. But how is one to explain the stabilization of a particle's coupling with a *vertical* surface? Such contacts result from Brownian motion

or inertial impactions. But what explains the continuance of their connecting relationship once initiated by contact? The usage of the term "mechanism" should perhaps explain why the particle/surface relationship continues and persists. It should reference the strength of the adsorptive bond that is established between the particle's and the filter's surfaces, albeit such information is rarely available. It is an explanation of these bonding mechanisms that will here be attempted in a more detailed if hypothetical manner.

The mechanistic forces are understood to be electrical in nature. The physical behavior of molecules towards one another is expressed through the electrical forces involved in electrostatics and electrodynamics. They are consequences of electrical-charges of various origins. Even the hydrophobic interactions that do not derive from obvious ion or dipole features that could initiate electrical interactions are, nevertheless, considered by some to be electrical in their influence. However complex, the adsorptive forces operational in the bonding of separate surfaces result from mutual electrical attractions. Almost certainly, other factors perhaps not yet known, are also involved. It is, however, the electrical interactions that will be considered here.

Filter Cake Formation

Cake filtration has been listed as a retention mechanism (Lee et al., 1993). Particles retained by a filter build a filter cake to a height commensurate with the fluid's extent of loading. Obviously, the greater the height of the filter cake, the greater its resistance to flow. The flow decay, the reduction in flow rate thus occasioned, depends upon the number, sizes and shapes of the constituting particles, and also on their hardness. The permeability of a normal packing pattern of discrete, hard silica-sand particles will better resist compaction. Cakes of softer, more gelatinous particles will more easily be deformed by pressure to undergo a loss in permeability. The many type particulates that are likely to be encountered will, thus, exhibit a spectrum of flow decays reflecting particle rigidity and shape.

Mathematical formulae have been developed by which different rates of decay may be interpreted in terms of the mechanisms by which the particles were assembled. In essence, the sites and manners of particle depositions can be deduced from flow decay studies. Given our paucity of knowledge concerning pore structures, the conclusions reached are at best highly conjectural. Nevertheless, they are not totally devoid of significance. Differences in the rates of flow decay are interpreted as resulting chiefly from sieve retentions or mainly from adsorptive sequestrations. The faster decays are believed to be the products of size exclusions; the slower rates are seen to result from adsorptive captures. Flow decays marked by intermediate rates are taken to indicate mixtures of the particle arresting mechanisms.

The logic involved has the larger particles rather completely blocking the flow-restrictive areas of the pores, whether at the pore openings at the filter surface or within the passageway itself. The latter occurs when the particle is small enough to enter the pore, but is large enough to block it internally. Particles too small to precipitously block the liquid flow adsorb to the pore surfaces such as the pore walls. The adsorptive captures serve to clog the pores, eventually to block them by bridging. Their accumulation is a function of time. Thus, the slower rates of reductions in flows characterize the adsorptive sequestration operations. The more sudden blockages are ascribed to the sieving mechanism. Mixtures of both mechanisms of particle removal are signaled by flow declines whose rates are between the two extremes. Such intermediate rates of decay depend upon the particle size proportions of the mixture.

THE MODELING OF PARTICLE CAPTURES

The differentiation between particle retentions by sieving and by adsorptive sequestration may be sought through mathematical modeling. It is assumed that bacterial retention is the controlling occurrence; the one leading to the eventual blinding of the filter. Particle retention takes place to build an ever increasing filter cake. Its limiting permeability decreases as it builds over time. This introduces a growing hydrodynamic resistance to flow at constant pressure. The mathematical treatment leads to a distinction between the two capture mechanisms. It distinguishes between the rate of change in flows that eventuate from sieving and from adsorption. Size exclusion is assumed to cause pore blockage rather rapidly by particles too large to enter pores. The same results from particles small enough to enter pores, but large enough to block the constricted areas within them. Adsorptive sequestrations are also presumed to take place progressively. The accumulation of smaller particle within pores serves increasingly to clog them by reducing the total pore volume. The end result is a clogging of the passageways more slowly to the point of complete blockage. The surface adsorption of smaller particles followed eventually by bridging of the pores also occurs. This differs from the more precipitous blockage of cake formation by the larger particles. The relevant factors are expressed mathematically by Ruth et al. (1933) in the case of pore blockage. The mathematics pertaining to particle adsorption and pore clogging were elucidated by Hermans and Bredee (1936).

The mathematical treatments, as said, embody certain assumptions. Where bacterial removal is the concern, it is assumed that bacterial retention is the eventually cause of filter clogging and blocking. Also assumed is the non-compressibility of the filter cake, an assumption that is rather suited to more rigid particulates than bacteria. The assumption made from time-to-time that sieve retention is a solely a surface phenomenon can be challenged. Thin though the membrane is, particle retention need not necessarily be confined to the filter's outside surface. It may occur upon the pore walls to cause clogging.

This type of flow decay or flux decline study is performed using constant-pressure conditions. Plotting is periodically made of the volume or throughput as a function of time. Flux decline during filtration will be a consequence of any retention mechanism, but will follow different time-volume relationships depending on the mechanism governing the filter's clogging and/or blocking. Given its numerous assumptions and several uncertainties, the mathematics involved in interpreting the plotting of the flow decay data may lead to non- rigorous results. However, they are not without significant implications.

Bowen et al. (1976) derived and applied mathematical equations to differentiate between the effects of size exclusion and adsorption. The assumptions inherent in their approach is more fully treated in the final section of this chapter.

For Sieve Retention

The most commonly used model (Ruth et al., 1933) for bacterial filtration by sieve retention is that of a porous matrix whose pores are smaller than that of the organisms. In such a situation, the bacteria create a filter cake that grows in thickness as the filtration progresses. The cake will add a resistance to the flow at constant pressure, the instantaneous rate of filtration at time t, $J_V(t)$, and the total volume of filtrate up to time t, $V(t)$, will change in a disproportionate manner as a function of time. Assuming an incompressible cake and a constant pressure differential across the filter, the relation is:

$$\underline{t} = \underline{k}[V(t) + 2V_f V(t)]$$

where V_f is the volume of filtrate required to produce a change in total resistance equal to that of the filter; K is a "filtration constant" that depends on pressure, ΔP; viscosity, η; filter area, A; cake resistance, R_c,; and particle concentration, C, in the following way:

$$K = 2A^2P/\eta CR_c$$

From these simple relations, it follows that a plot of $t/V(t)$ versus $V(t)$ should yield a straight line with a slope of K and an intercept of KV_f. Such a plot constitutes a first verification of the sieve retention, or, as also called, the surface retention model. The term surface signifies the particle's arrest at the restrictive pore site, whether at the filter surface or within the pore.

For Adsorptive Sequestration

Adsorptive capture, whether of a particle or of a soluble or near-soluble entity from the solution, involves the entry of that particle, viable or otherwise, into the pore channel. In these situations the particle being adsorptively retained is smaller than the filter's pore. (Even though the pore entrance is larger than the organism, sieve statistics dictate that a substantial fraction of the bacteria will be excluded, approximately 99.9% for pores 10% larger in diameter than the particle.) The convective flow situation existing within the pores will tend to transport the entering particles through the membrane. However, the attractive forces, when sufficient in strength, act between the bacteria and the pore walls against the convective flow, and promote interception of the particles (Fig. 11). In terms of the model, all these forces are combined and treated as a first-order reaction between the particle and the wall.

Each particle "reacting" within the pore cavity, that is, being adsorbed, reduces the total pore volume. Where k is a filtration constant related to the internal pore area and the particle concentration, the equation expressing the adsorptive model of flux decrease is:

$$\frac{t}{V(t)} = \frac{kt}{2} + \frac{1}{J_v(0)}$$

or

$$\frac{1}{J_v(t)} - \frac{1}{J_v(0)} = kt$$

where k is a filtration constant related to the internal pore area and the particle concentration.

A plot of $t/V(t)$ versus t should yield a straight line with a slope of $k/2$, and such behavior constitutes a test of the model, wherein the particles are retained within the pores by adsorption. Smaller particles must first gather to bridge the choke points of the pores (Fig. 12). The particle accretions build a filter cake that is permeable until the differential pressure is exceeded by the pressure drop resulting from the cake's resistance to flow. The permeability of the cake can be foreshortened by its compaction under increased pressure. The onset of impermeability depends upon the structure of the cake and its resulting compressibility as determined by the particles' sizes, shapes, numbers, and pattern of packing.

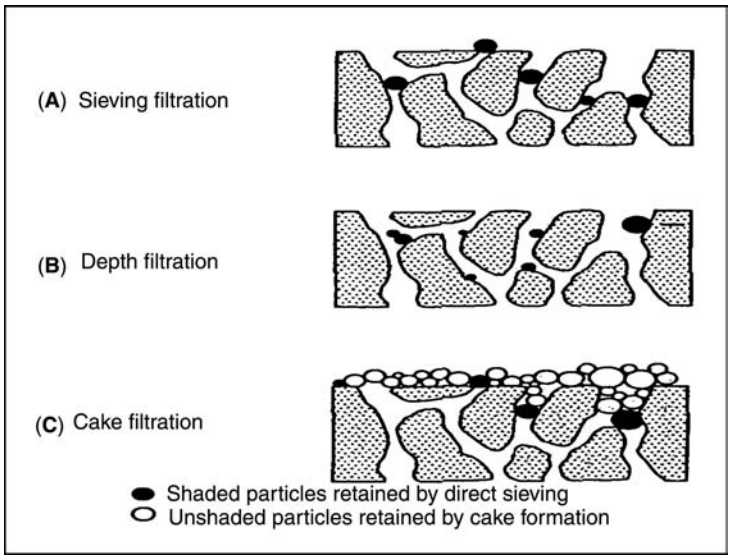

(A) Sieving filtration
(B) Depth filtration
(C) Cake filtration
Shaded particles retained by direct sieving
Unshaded particles retained by cake formation

FIGURE 12 Particle capture mechanisms in liquid filtrations. *Source*: From Lee et al., 1993.

Straight Line Plotting

The data reported by Wallhäusser (1979) (Table 1) were plotted in accord with the sieve retentive model and also with the adsorptive model. Figure 13 shows a straight line indicating the particle capture to be the result of an adsorptive mechanism. The non-linear line resulted from plotting the data for sieve retention. This signaled that sieve retention was not the mechanism at work.

The data obtained by Tanny et al., (1979) were plotted for adsorptive captures of *B. diminuta*. Both 0.2/0.22- and 0.45-μm-rated membranes were used in separate but

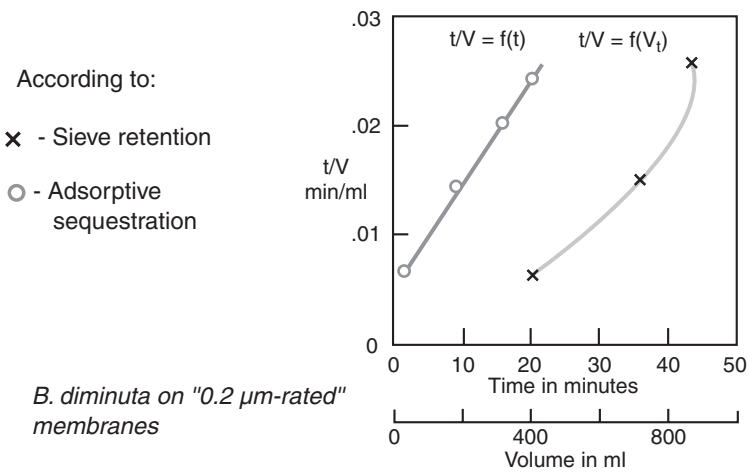


FIGURE 13 Plotting of Wallhäusser's (1976) data. *Source*: From Tanny et al., 1979.

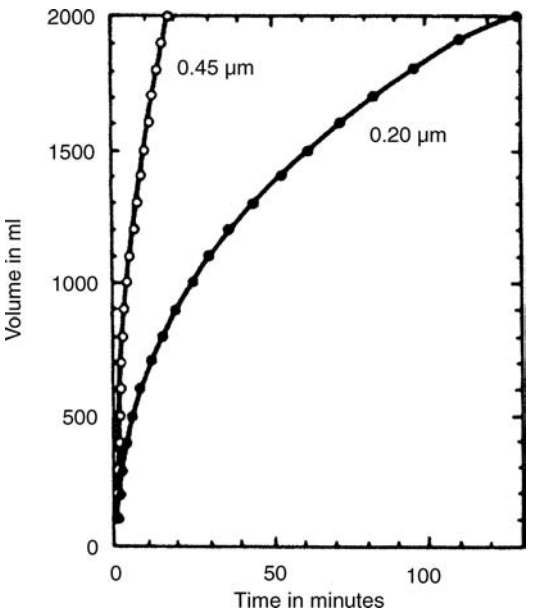


FIGURE 14 Flow decline data compiled for 0.2 µm-rated and 0.45 µm-rated membranes. *Source*: From Tanny et al., 1979.

identical tests. The straight line obtained for the 0.45 membrane denoted that adsorptive capture was operative. The non-linear, curved line signified that adsorptive interactions did not govern *B. diminuta* retention by the 0.2/0.22 membrane (Fig. 14). The results forthcoming from the plotted data were what might have been predicted from the relative pore size ratings and the size of the organisms.

An interesting set of curves resulted from challenging 0.45-µm-rated cellulose acetate membranes with different quantities of *B. diminuta,* namely, 1×10^4 and 1×10^5. The lesser challenge showed the straight line plot indicative of adsorptive captures. The early reaches of the 1×10^5 challenge likewise yielded a linear curve of the same significance. However, as more organisms were filtered from the challenge stream, the line began deviating from its straight course (Fig. 15). This signaled a departure from

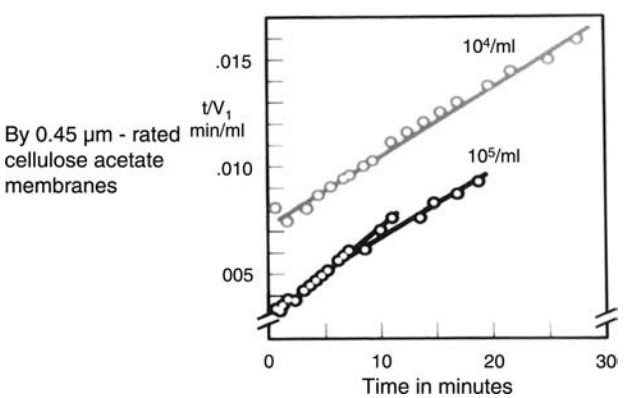


FIGURE 15 *B. diminuta*, retention at two different concentrations. *Source*: From Tanny et al., 1979.

the mechanism of adsorption. Tanny et al. (1979) rationalized the results as follows: The early removal of the *B. diminuta* from the 1×10^5 challenge was accomplished by adsorptive sequestration. This resulted in a clogging of the filter pores. Thereafter, the organism removal became an exercise in sieve retention.

One may conclude from the above examples that the mechanism of retention responsible for organism removals in particular filtrations may indeed be identified by a proper plotting of flow decay data.

Retentions by Particle/Filter Contacts

Gases, like liquids, operate largely by size exclusion in being separated from their particulates. Adsorptive sequestrations can come into play when suspended particles are given motion that culminates in their encounter with a filter surface. The several ways in which particles directly encounter filter surfaces, such as through inertial impactions, Brownian motion, gravitational force, may also be considered retention mechanisms. The particles become fixed to the membrane surface by electrical attractive forces, subsequently to be explained. These constitute the adsorptive bonding between the surface of the impacting particle and the surface of the filter.

Gravitational Settling

Such gravitational impacts can come about when particles suspended in a flowing fluid atop a filter are heavy enough to settle out in response to the force of gravity in accord with Stoke's Law: The larger the particle, the faster its settling rate.

Inertial Impaction

The inertial impaction of a particle upon a filter surface can occur when the fluid bearing the particle changes its direction of flow as it is deflected into and through the filter pores. The inertia of the particle may continue it on its original path to collide with the filter surface where adsorptive forces can cause its arrest (Fig. 16). This inertial force depends directly upon the mass of the particle, and the square of its velocity. It is, therefore, more important with heavier particles. The inertial force is attenuated by the viscosity of the fluid. Consequently, it can be influenced by temperature which is inversely related to viscosity. For this reason it is less effective in liquid than in gaseous contexts.

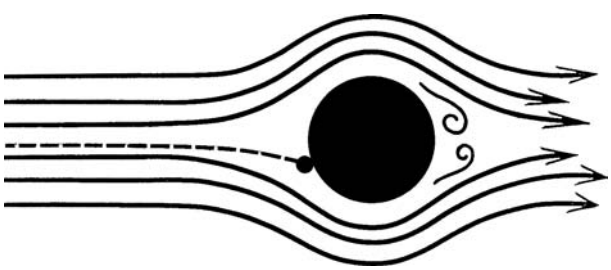


FIGURE 16 Inertial impaction. *Source*: Grove and Davelose. J Filtration & Separation, 1982.

Brownian Motion

Smaller particles, less heavy, are less influenced by inertia. However, they are more affected by Brownian motion wherein they are vectored from the fluid pathway to the pore surface by collisions with the fluid's molecules. The result is retention of the particles by the filter. The nature of the bonding that adheres the one surface to the other, the actual mechanism, will shortly be elucidated. At all temperatures above absolute zero the various sections of all molecules are in constant motion; the various bonds being flexed, rotated, stretched, etc. The higher the temperature, the greater the amplitude of the molecular motion. The significance of absolute zero is that only at that temperature or below is all molecular movement frozen. In their frenetic activity, the fluid molecules collide, perhaps repeatedly, with suspended particles. The latter are impelled to new directions of travel within the fluid stream. As a result of their induced random and erratic movements the buffeted particles have opportunities to encounter pore surfaces and to become attached thereto by electrical forces we have yet to describe. This is the nature of Brownian or diffusional interception (Fig. 17). It is favored by the small size of the particles, and by the lower viscosities of the suspending fluids. Thus, it is more important in gas rather than in liquid filtrations.

RELATIVE RETENTION EFFICIENCY OF GASES AND LIQUIDS

All molecules and their component atoms and linkages are, under normal conditions, in constant motion. Their bonds flex, stretch, and rotate in response to temperature. The motion diminishes with decline in temperature, and ceases only at absolute zero. Indeed, this is a definition of absolute zero. Thus, the molecules of a fluid medium are in constant collision with one another, and with any particles suspended therein. The mean free paths of the molecules in motion are less restricted in gaseous contexts than in liquid because of the relatively fewer interruptions by collisions with other molecules, the gas molecules being more widely separated from one another than molecules in liquids. Therefore, Brownian motion and inertial impactions that vector suspended particles to filter surfaces are more effective in gases than in liquids. The attenuation of particle movements caused by the viscosity of the suspending fluid that reduces the opportunities for encounters between filter surfaces and particles is an impediment to adsorptive bonding. This accounts for the findings of Megaw and Wiffen (1963) that relatively large pore-size rated filters are capable of removing particles some two magnitudes smaller than their ratings with very high efficiencies. These investigators showed that 0.05 μm particles are retained by 0.8-μm-rated membranes. Leahy and Sullivan (1978) challenged equivalent

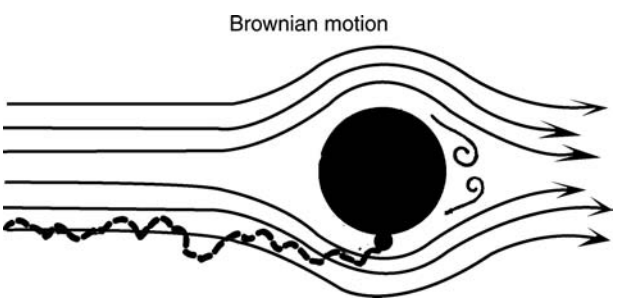


FIGURE 17 Brownian or diffusional interception. *Source*: Grove and Davelose. J Filtration & Separation, 1982.

filters in liquid media using organisms larger than the Megaw and Wiffen particles by a factor of 10. Despite the larger sizes, the particle capture efficiencies in liquid were magnitudes lower.

Most Penetrating Particle

For both inertial impactions and Brownian motion the consequences of the particle's connecting with the filter surface is attenuated by the viscosity of the liquid medium; not so in the case of gases with their low viscosities. These impact mechanisms are, therefore, of greater influence in removing particles from gas streams than from liquids. The particle's mass is very important to the effectiveness of inertial impactions; the Brownian motion, diffusive by nature, is far less influential on these larger particles. The inertia of the smaller particles is too minor to have a significant effect. According to Liu et al. (1983), it is the diffusional interceptions of these smaller particles, the result of Brownian motion, that is responsible for the high filtration efficiencies of air filters. The rate of particle capture is inversely proportional to the square root of the particle's diameter. This is especially true for particles below 0.3 μm in size, particularly in dry air, and at low air stream velocities. The opposite is true for the larger particles because their mass, especially as multiplied by their squared velocity, has so important an effect on their inertia. As the particle mass decreases, the inertial impactions diminish in influence. It turns out that particles of about 0.3 μm in size, modified somewhat by their velocity, are the least retained by either of these two types of impactions. Therefore, particles of this size are the most likely to penetrate an air filter. This, then, by definition, is the size of the most penetrating particles (Fig. 18).

Modifications in filter design are necessitated by the increases in the air stream velocities that are required in specific applications. At higher velocities, smaller particles

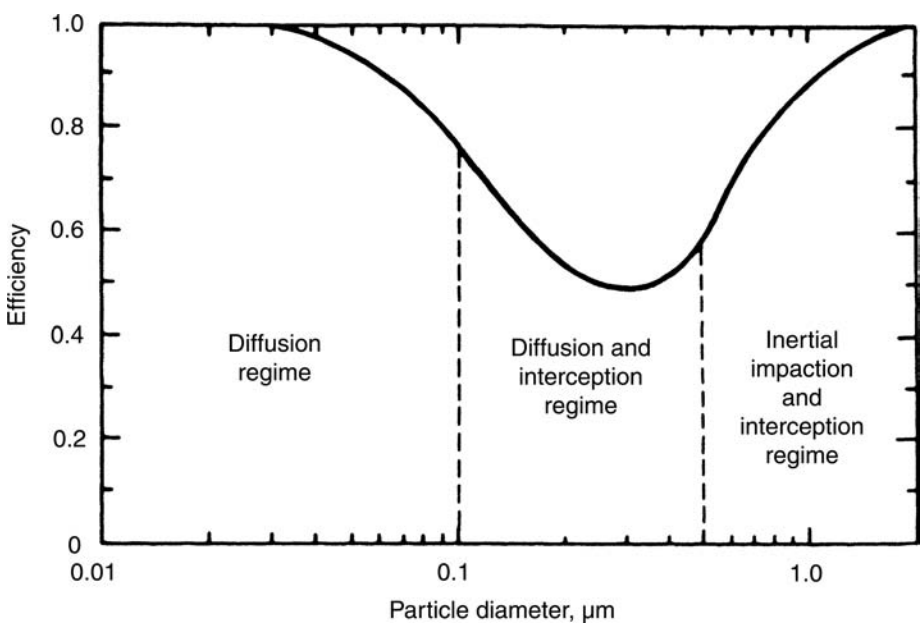


FIGURE 18 Filter efficiency as a function of particle size. *Source*: From Liu et al., 1983.

assume a higher inertia, the product of their mass and the square of their velocity. This decreases the size of the most penetrable particle. The 0.3-μm particles, their inertia enhanced by the increase in velocity, are no longer the least possible to capture. That designation passes to smaller size particles. Therefore, filters intended for such applications as vent filters need to be designed accordingly.

The Adsorptive Bonding

The conjoining of a particle and filter surface by way of size exclusion need not necessarily result in an organic union between the two. Their adjacency may involve nothing more than the particle having been deposited passively at the filter surface by the flowing fluid. Aside from the accidents of geometry and gravity, other ways of two surfaces interacting involve some exercise of a bonding force. By the term "adsorptive sequestration" the authors means to characterize whatever forces result in a bonding strong enough to remain viable following the separate surfaces having made contact. Depending upon semantics, one can choose to describe the situation as resulting from different types of adsorptions, or one can speak of the one adsorption mechanism and its several manifestations. Either view is acceptable to the authors.

The theme of this writing is the coming together of two surfaces, namely, that of a particle (more explicitly of an organism), and that of a filter in a union that is the result of bond formation. The situation is that of a particle carried by a flowing fluid stream into contact with a fixed-in-place filter. The particle remains attached to the filter, thus fulfilling the purpose of the filtration. Our inquiry pertains to the origin and nature of the bonding forces. The particle removal is complex in that there are conflicting forces simultaneously in operation: One produces a mutual attraction between the two surfaces; the other exerts a mutually repulsive influence. The desired interaction requires the attenuation of the stronger repulsive force to enable the dominance of the weaker attractive action.

Briefly, to be elaborated upon later, the repulsion arises chiefly from strong charges of the same sign, namely, the coulombic forces. The genesis of the attractive forces are several. Zydney (1996, Chap. 9) list one source as being quantum-mechanical in nature. The second is electrostatic in its action. It derives from interactions between fixed charges and/or fixed dipoles. The third type of attractive force is the product of molecular polarizations caused by induced dipole–induced dipole interactions. These are the so-called dispersion forces also known as the London–van der Waals (VDW) forces. The respective forces differ in their origins; more importantly, they differ in their strengths. The formation of the adsorptive bond requires the balancing of the strengths and directions of the several operating forces. This determines the distance at which the influences of the attractive forces become effective. It is known as the Debye length. The means whereby the Debye length is reduced to the point where the attractive forces prevail is the addition of salts to achieve a high ionic content. At its culmination is the adsorptive bonding of separate surfaces, such as of an organism and a filter surface.

The mechanism responsible, in its successive stages, for this achievement was first elucidated in the destabilization of colloidal systems.

Colloidal Destabilization

In its simplest form the colloidal state is a suspension of discrete particles that resist settling out even over long periods of time. Colloids, of whatever composition, consist of

particles from 0.001 to 1 μm (10^{-7} to 10^{-4} cm) in size, too small to be visible under an optical microscope. Colloidal particles are too small in size, (and hence in mass), to be responsive to Stoke's Law.[a] Colloidal particles are subject to Brownian motion. They are given erratic movements through collisions with the ions in solution. This helps prevent their settling out. The colloidal particle has a large surface area. This encourages the adsorption of ions and the concomitant acquisition of electrical charges. (Colloidal charges can also result from the ionization of molecules on the surface of a particle, or from the dissolution of ions from the solid into the liquid state.) Since like-charges repel, and since all the particles constituting a colloid bear the same charge, the discrete particles repel one another and do not agglomerate to form a sediment.The adsorptive joining of one colloidal particle to another involves the same forces of attraction that regulate the adsorption of dissolved molecules by membranes, or of organisms from their suspensions. Both attractive and repulsive forces manifest themselves simultaneously. The attractive forces have only a short-range effectiveness; the repelling forces are stronger and operate over a longer distance. The adsorptive process is essentially one of overcoming the repulsive, long range, forces. A most important consideration, then, is the distance separating the molecules or particles being adsorbed and the adsorbing sites on the filter. It is over this distance, the Debye length, that the attractive forces between the separated surfaces must operate in order for them to come together to form the adsorptive bond. This is not achievable at great distances. However, at shorter distances the attractive forces, whether of hydrophobic or more overt charge-related origins, prevail. Increasing the ionic strength of the suspending liquid by the addition of salts interposes ions between the charged particles. This reduces the charge density, the zeta potential, and the distance over which it has influence. The attractive forces are enabled to assert their powers over the reduced distance. The solid surfaces of the particles then undergo adsorptive bonding with one another, and agglomeration results.

An intriguing view of the effects of the zeta potential, the measure of the electrokinetic effect, vis a vis colloids is given by Pall et al. (1980). These investigators point out that colloidal suspensions are stabilized when their particles are endowed with net surface charges of similar sign in the magnitude of 30–40 mV or more. The mutually repulsive forces then suffice to repel the particles from one another. The double layer distance is then large enough to frustrate the shorter range attractive VDW forces. Therefore, no flocculation occurs, and the colloidal dispersion is stabilized. Below about 30 mV the double layer extent shortens, and the zeta potentials begin to reflect the growing involvement of the attractive secondary valence forces. Marshall (1992) considers the critical Debye length to be from 10 to 20 nm, at which point "... long range (sic) van der Waal attractive forces can exceed 'the' electrical repulsion forces ...." Over and at the zero charge level, attraction dominates and flocculation occurs: The colloid becomes destabilized.

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[a] Stoke's Law relates to the settling of suspended particles. It reflects two factors: The density differences between the particle and fluid, and the size of the particles. The greater the density differences, the faster the settling. The larger the particle, the faster its settling rate. The gravitational force acting upon a particle to cause its settling varies with the square of the particle diameter.

$$V_{Stokes} = (d_1 - d_2)a^2 g/V = \text{settling velocity}$$

where a is the particle diameter, g is the gravitational constant, d_1 is the particle density, d_2 is the liquid density, and η is the liquid viscosity.

Consideration will now be given to the DLVO theory, in its development from the established Debye-Hückel theory accounting for the electrical charge phenomena that govern the filtrative removal of particles from their suspensions. The designation DLVO derives from the initials of the theoreticians' family names: Derjaguin, Landau, Vervey, and Overbeek.

Electron Sharing and Electrical Charge

In an oversimplified view, an atom consists of a very dense nucleus that comprises its mass and is positively charged because it contains positively charged (+) subatomic particles. Surrounding, but relatively far removed, are concentric rings of negatively (–) charged electrons. Often referred to as the "electron cloud, the implication is of continuous movement of the electrons at such speeds as to blur their momentary positioning. The electrons in the outermost shells are the least firmly bound. They are characterized by having the highest quantum numbers. They are the valence electrons whose activities are involved in chemical reactions between atoms. The remainder of the atom is known as the "core" or "kernel". Atoms are uncharged, possessing just enough electrons (–) to neutralize the nucleus' (+) positive charges. Each ring is limited to an exact number of electrons; usually eight except for the lighter elements, most notably hydrogen. The outermost ring is mostly incompletely filled. An atom in forming a molecule will completely fill or empty its outermost shell by transferring, (accepting or donating), electrons to another atom that is under the same compulsion, or by sharing the two bonding electrons with another atom to the same purpose.

Since the electron, by convention, is negatively charged, atoms that come to possess more electrons than they do in their neutral state, are labeled as being negatively charged. If they contain fewer electrons than in their neutral state, they are designated as being positively charged. Atomic and molecular entities react to one another in response to their plus or minus electrical status. It is generally comprehended that opposite electrical charges attract and bond to one another, whereas like-charges mutually repel.

Bond Types

Electron transference or sharing occurs as a response to valence requirements. The formation of a valence bond lowers the energy of the formed structure, making it more stable. The ionic bond that is formed by the donation of an electron by one atom and its acceptance by another is strongly charged electrically. Being strong, it exercises its influence over relatively longer distances. It is often called a primary bond. The covalent bonds fashioned by electron sharing are not, unlike the ionic bonds, structurally and inherently charged. Although not so strong as the ionic bond, the covalent bond too is considered a "primary bond." These two types of bonds, namely, the ionic and covalent, are the valence bonds. They differ from the bonding that is caused by the partial sharing of electrons. Although respectively called "primary" and "secondary" or "strong" and "weak," or even "chemical" and "physical," these bonds cover an entire spectrum of strengths. There is perhaps "no sharp dividing line between the true bonds and the weaker interactions described in different terms" (Wheland, 1947).

The Ionic Bond

The ionic bond, as said, involves the complete transfer of an electron from one atom to another. It is characteristic of inorganic salts. They mirror the electrical charges that result

from the electron's change of location. Consider the union of a sodium atom and a chlorine atom. In the electron transfer described, the electrically neutral sodium atom, now bereft of an electron (–), becomes changed into a positive charged sodium cation. The neutral chlorine atom, having acquired an electron, is now negatively charged. It is now a chloride anion. The transfer of the electron that creates the ionic bond is total and complete. The electron whose transfer created the negative charge stays completely with the chloride ion. It is not shared with the sodium ion. The two oppositely charged ions interact on the basis of the mutual attraction of their opposite charges to create a molecule that is a salt, sodium chloride.

The molecular combination consists of an aggregate of positively charged sodium ions in lattice form juxtaposed to an assembly of negatively charged chloride ions in lattice form; the lattices being connected by the strong attractions of their opposite electrical charges. The two oppositely charged ion lattices are separated when the salt is dissolved in water, it being a polar medium. The water molecule carries partial positive charges on its hydrogens, and a partial negative charge on its oxygen atom. The orientation of the water dipoles in an alignment of plus-ends to minus-ends serves to moderate the full coulombic charges of the ions. This attenuation allows their separation. Nevertheless, although now separated, the ionic charges are strong enough to exert their attractive or repulsive powers over long distances.

This is in contrast to the weaker forces that result from the partial charges arising from the unequal sharing of covalent bonding electrons, or from other phenomena associated with covalent bonding (see the following section).

The Fuoss Effect

The positive effect of ionic strengths on adsorptive sequestrations is forthcoming from the field of water treatment and involves the adsorption of organic substances by activated carbon. This is greatly enhanced by the presence of calcium and magnesium ions. According to Weber et al. (1983), the adsorption of humic materials by activated carbon is pH-dependent and is influenced by the presence of inorganic ions in the solution (Fig. 19). Calcium is slightly more effective than magnesium, and divalent ions are more influential than monovalent ions by an order of magnitude; potassium ions are slightly more effective than sodium ions. The salutary effects of lower pH on increasing adsorption had previously been remarked upon by Schnitzer and Kodama (1966). A plausible explanation may derive from the Fuoss effect as discussed by Ong and Bisque (1966) and as advanced by Ghosh and Schnitzer (1979).

The Fuoss effect states that large polymeric electrolytes, such as derive from humic acids, exist in solution in a coiled configuration, as indeed do all polymers. Polymeric molecules increasingly unwind and extend themselves the diluter the solutions (Fig. 20). Increasing ionic strengths, to the contrary, promote the tightening of such coiling. The contractions of the humic acid molecules under the influence of higher ionic strengths have two adsorption-promoting consequences. The size of the polymer molecules decreases as they become progressively more coiled (Fig. 21). In the process, the folding of the polymeric chains increasingly confine their hydrophilic moieties, and more openly present their hydrophobic constituents, of which tryptophane, tyrosin, and phenylalanine are the most extreme. The first effect further increases the ease of interstice penetration; the second promotes hydrophobic adsorptions. Thus, the presence of ions such as hydronium, calcium, and magnesium promote the molecular coiling. Both the capacity for adsorption and the rate of adsorption are increased. This is significant because adsorption is rate-dependent.

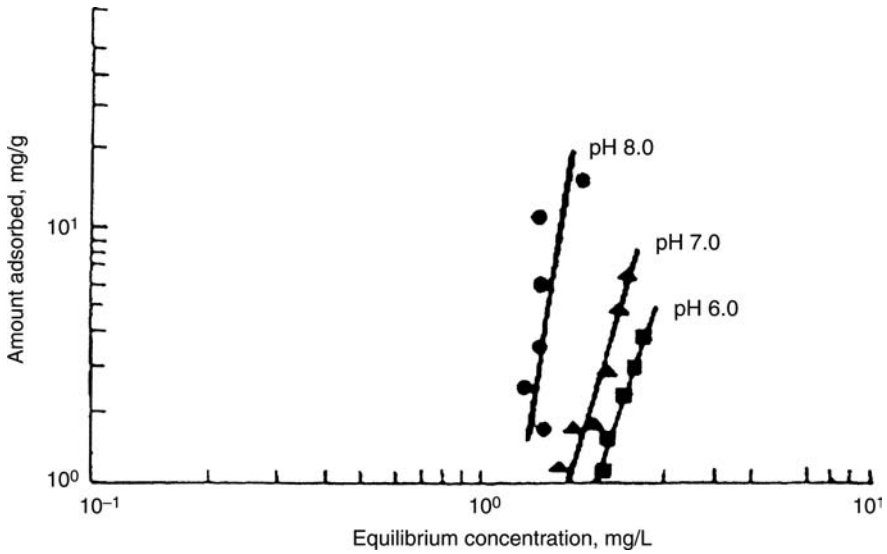


FIGURE 19 Adsorption isotherms for humic acid on carbon-effect of pH. *Source*: From Weber et al., 1983.

Evidence that the adsorption onto active carbon surfaces of organic materials derived from humic substances is promoted by lower pH was furnished by Weber et al. (1983) and by Schnitzer and Kodama (1966) and was stated also by Michaud (1988). Weber et al. 1983 found that the adsorption isotherm for humic acid on an activated carbon, although increased somewhat by going from pH 9.0 to 7.0, increases markedly when the pH is lowered to 3.5.

Endotoxin Adsorption By Ionic Interaction

As stated, the transfer of the electron that creates the ionic bond is total and complete. The resulting ions combine with ions of opposite charge. This type of bonding is used to remove endotoxin from solutions. The pyrogenic lipopolysaccharidic endotoxins are retained by membrane filters bearing positively charged functional substituents. These are polyamide membranes (nylon) whose molecular structures have been chemically

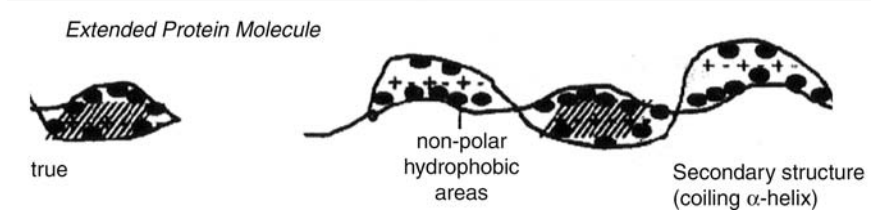


FIGURE 20 Polymer in dilute solutions. *Source*: From Mittleman et al., 1998.

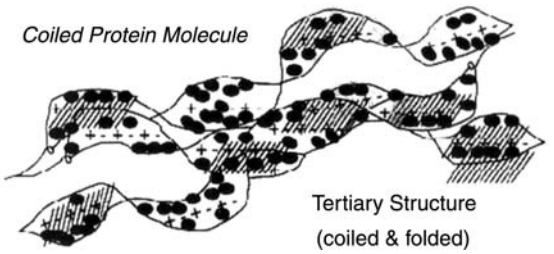


FIGURE 21 Polymer in concentrated solutions. *Source*: From Mittleman et al., 1998.

modified to yield quaternary amine groups affixed to the polymer chain. The quaternary-substituted nitrogen atom is ionic; it bears a positive charge. The adsorptive interaction involves the attractive forces operating between the positive charges on the membrane and the negatively charged surfaces of the endotoxin aggregates, which behave as anions at pH > 2 (see section on Endotoxin Removal by Hydrophobic Adsorption).

Carazonne et al. (1985) showed that charge-modified nylon membranes remove pyrogenic substances from solutions with an efficiency that depends upon the composition of the liquid. The endotoxins used were extracted from *E. coli* 055:B5. The removed pyrogens are firmly adsorbed. They are not released during continuation of the filtration process. However, the charged nylon filters exhibit a finite adsorption capacity; the pyrogen removal efficiency decreasing with the successive aliquote being filtered. The capture mechanism is charge mediated on a stoichiometric basis. As the positive charges become progressively neutralized, the capture efficiency decreases, and endotoxin breakthrough becomes more likely. At any stage of the filtration, the removal efficiency which reflects the encounters between the anchored positive ions and the endotoxin units, is very dependent upon the rates of flow, and the progressively diminishing number of positive charges. The higher the flow rate proportional to the remaining number of positive charges, the more likely it is that the removal efficiency will diminish. The endotoxin unit may exit the filter before the positive-charge site is collided with. Endotoxin breakthrough is the first indication of insufficient numbers of remaining charges, whether through their exhaustion, or too high flow rates. In sum, the main factors affecting this type of charge-retention are: the EFA, the total volume filtered, and the rates of flow as determined by the delta pressure. Other factors, such as pH, viscosity, and temperature also contribute to the final outcome.

To avoid the impracticalities of endotoxin breakthrough, two filtration units in series with a sampling port in between enables ascertaining, through the use of periodic sampling, when the upstream filter permits endotoxin penetration. At this point, the relatively unused downstream filter reassures against unretained endotoxin contaminating the final effluent. Replacement of the exhausted filter, and reversing the direction of flow through the filters enables the replacement filter to safeguard the purity of the effluent (Fig. 22). Validation is required to determine the time to exhaustion for the upstream filter. Evaluation of endotoxin break-through necessitates using the fluid product under process conditions.

Competition Among Ions

Carazonne et al. (1985) found that in the case of a deionized water containing pyrogen concentrations comparable to 12 ng of *E. coli* endotoxin, the adsorptive removal by the

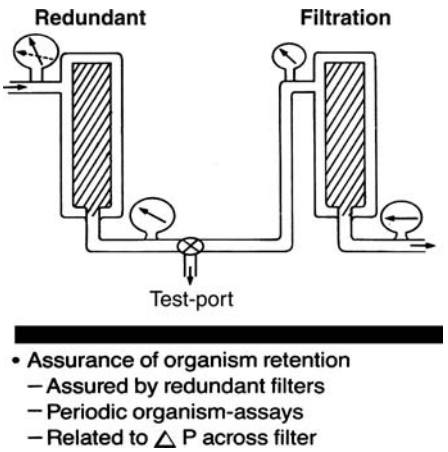


FIGURE 22 Safeguarding the purity of the effluent. *Source*: Jornitz, 2006.

charged nylon filters can be accomplished. Pyrogen removal from 5% glucose solutions is not interfered with. However, the presence of 2% peptone solution, at either pH 3.8 or 8.3, inhibits the removal of endotoxin from solutions. This bears a similarity to the experience of Bowman et al. (1963) who found that the protein penicillinase, by preempting the adsorption sites, interfered with the adsorptive removal of *B. diminuta* by non-charged membranes filters. Perhaps surprisingly, electrolytes, and specifically 0.9% sodium chloride solution prevent the removal of the endotoxin by the positive-charged membranes. To this should be added that the stoichiometric relationship between charge site and the endotoxin unit results in the loss of two positive charges when one sulfate ion, having a double negative charge, is neutralized at a single site. This very situation explains the charge reversal that these membranes may undergo, releasing their captured endotoxin in the bargain. Carazzone et al. conclude, "positively charged media are interesting, but need careful preliminary studies in order to define their suitability and operational procedures."

Fajan's Rule

It may be that there is a hierarchal order governing anionic attractions to the membrane's positive charges. If so, it may be similar to Fajan's Rule that governs ion-exchanges, namely: It depends on the charge density of the ion. This, in turn, is the ratio of the charge to the ion size. The greater the charge density of the ion, and the smaller the ion, the more closely it can approach the opposite, fixed-ionic-charge involved in the exchange reaction, and the greater its selectivity. However, in aqueous media the ion radius is not its isolated or crystallographic radius, but is rather that of its hydrated state. The smaller the isolated ion, the more closely it can approach water molecules and the more plentifully it can be hydrated by them. Thus, the proton, the smallest of all cations, becomes surrounded by many waters of hydration, and conversely acquires the largest radius. The potassium ion is larger crystallographically than the hydrogen ion, but is increased is size by only 5 or 6 waters of hydration. Its effective radius in its hydrated state is, therefore, smaller than that of the hydrated proton. That ion, being relatively small crystalligraphically, acquires a large skirt of water molecules.

This finds reflection in the selectivity of the cation-exchange reactions. The larger the hydrated ion, the weaker is its bonding, and the more easily is it exchanged. In the case of anions, the hydroxyl ion, being relatively small crystallographically, acquires a large skirt of water molecules. It is the largest anion in its hydrated form, and is, therefore, the least preferred in anion-exchange reactions. This would also explain the effect of pH on the removal of endotoxin by charge modified membranes.

The Covalent Bond and Partial Charges

The covalent chemical bond is formed by a sharing of two electrons by two atoms. It is typical of organic molecular structures. The covalent bond forms when the electrons in the outer shells of the electron clouds of the two constituting atoms overlap in a complementary fashion. The overlap enables the electron-sharing that satisfies the fulfilling of the outer shells of both atoms. This establishes a bonding that connects the two atoms. (By contrast, strong repulsive forces result from incompatible electron cloud overlapping.)

The bond strength depends upon the two particular atoms involved, as does the degree of their sharing the bonding electrons. The sharing need not be equal, the propensity of different atoms for attracting electrons not being the same. Atoms are neutral in charge. An electron, by convention, is negatively charged. Therefore, the atom acquiring the greater share of the two bonding electrons than the one it contributed takes on a negative charge. The partner atom with its smaller share assumes a partial positive charge. The partial charges attract their opposite partially charged equivalents present in other molecules. Being only partial in their extent of charge, their attractions form bonds that are relatively weak. Their force extends over lesser distances, and when in opposition to repulsive forces arising from full charges are easily surpassed. Thus, they are called "secondary" bonds. However, they are important factors in adsorptive bonding such as are operative between organisms and filters that culminate in organism removals. (The symbol for the partial charge is the lower case Greek letter delta, Δ.)

Polarization and Dipoles

Molecular structures possessing dielectric properties manifest through the magnitude of their dielectric constant how much their presence will reduce the strength of an electric field. The polarization of dielectric materials is managed by way of an external electric field. The molecular polarity arising from the partial or unequal sharing of bonding electrons, or from other polarizing effects, results in a number of bond types. This includes bonds arising from van der Waal forces, and also from dipole structures, the most important of which are the hydrogen bonds or H-bonds. Polarity can be inherent in the molecular structure or can be induced by outside charge influences. Normal fluctuations in an atom's electron-cloud density cause instantaneous but constantly altering dipoles in that atom.

Dipoles are neutral molecules characterized by permanent and separate polar sites of positive and negative charges that do not coincide. They are characterized by this unsymmetrical arrangements of electrical charges. The dipole moment is a measure of the polarity of the molecule. It is defined as the distance between the charges, multiplied by the magnitude of one of the charges.

Their significance is that their induced polarity, in turn, induces dipoles into surrounding molecules. The VDW forces are of this character. Being induced dipoles,

induced by induced dipoles, they act between transient dipoles on separate molecules, not on permanent dipoles. Albeit effective at only short distances, they are very important influences in adsorption bonding at sufficiently short Debye lengths. The polarization of an isotropic dielectric is directly related to the strength of the external electric field that is its cause (Gabler, 1978, Chap. 4).

Polarization results from the alignment of permanent dipoles and /or induction of dipoles in the affected molecules or atoms. Permanent dipoles undergo better alignment. Moreover, the molecule's plus and minus charge centers are separated further. Larger induced dipoles result.

As stated, the several electrical forces differ in their origins, whether of valence or of different degrees of electron sharing, etc., and, thus, find expression in a spectrum of strengths. The chemical or valence bonds are the stronger. The weaker bonds, more easily disrupted, are the ones represented in adsorptions. Although weaker than the chemical bonding, the consequences of their effects are often profound, and include the many manifestations of hydrogen bonding, such as are important in protein chemistry. Both arise from particular arrangements of electrons and from the electrical charges that result. The very surface interactions that are operative in the adsorptive sequestration of organisms apply also to the agglomeration of colloidal particles, and to the fouling by proteins of filters, the latter by hydrophobic adsorptions.

The Dipole Structure

Molecules may be charge-neutral overall but may be complex enough to simultaneously contain positive and negative sits, whether ions or partial charges. As stated above, the partial charges leading to interactions can arise from several sources. In polar molecules, fixed dipoles may represent the finite distance that exists between the centers of positive and negative charged functional groups. An unequal sharing of electrons may be induced in a neutral molecule by the proximity of a dipolar molecule. As a result of this polarization, the molecule with the induced dipole will by its electronic imbalance be able to exercise its partial-charge influences on other neutral molecules, etc. An even greater polarization or electron-pair dislocation leading to a greater partial charge would be induced in an heretofore electrically neutral neighbor by the full electrical charge of an ion. At the other extreme, as will be discussed, VDW forces are hypothesized to be induced dipoles, induced by induced dipoles. They are instantaneous dipoles that average over time to zero. These are weak but significant electrical forces that are considered responsible for the charge interactions (adsorptive connections) between molecular structures that possess no obvious polar features. A common interaction is one between two bipolar molecules, whether of fixed structural origin, or induced. The hydrogen bond is an example. Following are some examples of the importance of hydrogen bonding.

The Hydrogen Bond

The hydrogen bond or H-bond is an important feature in the structure of water. What is known about the structure of water derives from studies involving such arcane subjects as neutron and X-ray absorption spectroscopy, and X-ray Raman scattering. More recently, time-resolved infrared spectroscopy has been utilized to determine the equilibrium position of the oxygen and hydrogen atoms on the attosecond (10^{-18}s) time scale by studying the time variations of vibrational frequencies. Comprehension of the hydrogen bond, (H-bond) is gained from studies of water in both its liquid and solid states using such esoteric techniques.

The hydrogen bond arises from a dipole/dipole interaction. It is the most important of such interactions. The water molecule, H_2O, consists of two hydrogen atoms each bonded to the same oxygen atom. The nucleus of the oxygen atom pulls the bonding electrons more strongly to itself and away from the hydrogen atoms. The bonding is not disrupted, but the bonding elements become partially charged. The unequal sharing of the electrons makes the electron-richer oxygen partially negative, and the proportionately deprived hydrogen atoms partially positive. This creates the $O^{-}H^{+}$dipole (Tanford, 1980, Chap. 5). There are two hydrogen atoms originating from two different water molecules that connect to a single oxygen atom of one of those molecules. One hydrogen atom is of the pair chemically bonded to the oxygen atom to comprise the water molecule. The other forms the hydrogen bond that bridges one water molecule to the other. Interestingly, the two hydrogens are not equidistant from the oxygen atom. The one attached by valence forces to the oxygen atom is at a distance of 1.00 ä from it. The H-bonded hydrogen is 1.76 ä apart from that oxygen. The oxygen atoms of the two interacting water molecules are 2.76 ä apart, while the two nearest non-hydrogen bonded oxygen atoms are 4.5 ä distant from one another. The chemically bonded hydrogen is more closely attached; perhaps an indication of the relative strengths of the two types of bonds.

The attractive forces of the oppositely signed partial-charges decrease rapidly with the distance between the dipoles. Only the proton (hydrogen atom, of atomic weight 1) is small enough to approach the electronegative oxygen atom closely enough to establish the H-bond. Moreover, the electromagnetic associations involving hydrogen atoms in dipole arrangements are only strong enough to be formed with the most electronegative elements, namely, fluorine, oxygen, and nitrogen in decreasing order. The H-bond strength is on the order of 4.5 kcal/mol; the range being from 2 to 10 kcal/mol. The covalent O–H bond has a strength of 110 kcal/mol (Zydney, 1996). Typically, the covalent bond strengths is about 100 kcal/mol, approximately the same as an ionic bond in a crystal of NaCl (Gabler, 1978, Chap. 5) Nevertheless, the H-bond, although weak in its energy of attraction, figures significantly in many fields of chemistry, and has importance especially in protein chemistry. The water molecule is tetrahedral in shape. The molecules of water in its solid (ice) state exist as tetrahedral hydrogen bonded structures. Much of this ordered form persists even in the mobile liquid. Each of the tetrahedral corners holds either a pair of electrons or an hydrogen atom. Each of the partly positive hydrogen atoms of one water molecule can form a hydrogen bond with a partly negative oxygen atom of each of two different water molecule. This commonly accepted view based on the interpretations of neutron and X-ray diffraction patterns holds that water molecules have the capacity to hydrogen bond with each of four other water molecules, but that fewer than that number are simultaneously present. This process, repeated throughout the water volume, creates an interconnected molecular network that includes H-bonded rings and chains of dynamically altered arrangements (Fig. 23).

Effect Upon Vaporization

Consider its effects upon the physical properties of various substances. As a general rule, the smaller a molecule, the more easily it is vaporized from its liquid state. That is, if it is a liquid, it has a lower boiling point. Conversely, if it is a gas, it requires greater pressures to compress it into a liquid. On this basis three rather simple molecules can be compared for assessing the influence of hydrogen bonding on physical properties. Hydrogen cyanide, HCN, has a molecular weight of 14; hydrogen sulfide, H_2S, has a molecular weight of 34; and water, H_2O, has a molecular weight of 18. In conformity with their

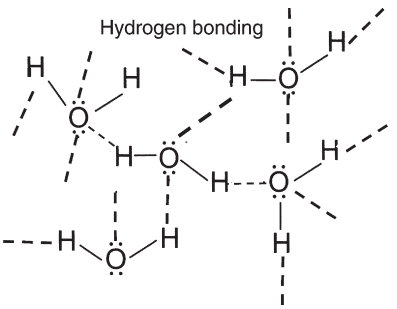


FIGURE 23 Water molecules. *Source*: Courtesy of Capitola Presentations.

mass, H_2O should have the lowest boiling point; HCN should be next; and H_2S should be the least volatile. However, water boils at 100˚C at atmospheric pressure; hydrogen cyanide, at 26˚C; while hydrogen sulfide boils at (minus) −59.6˚C.

In vaporizing so readily (at minus 60˚C), H_2S fits the rule that so simple a molecule should easily exist in the gaseous state at room temperature. HCN, being of a lower molecular weight, would vaporize even more readily were it not for the hydrogen bonds that form between the H of one HCN molecule and the N of another. The molecules joined by the hydrogen bonding comprise a larger mass, and, therefore, volatilize more slowly; heat being necessary to first break the H-bonds. The hydrogen bonds that are established among water molecules are dipolar associations of the H of one water molecule with the O of another. These are stronger than the dipolar H to N intermolecular bonds of HCN. Therefore, water requires the more considerable heat of 100˚C temperature to break its hydrogen bonds enough to become the gaseous molecule it should be at a molecular weight of 18. Even at that, much of the vaporous form of water is as a dimer, a combination of two water molecules joined by a single H-bond. Thus, hydrogen bonding accounts for many of the properties of water, such as its wetting and solution properties, etc. Its density/temperature relationship is among them.

Density of Water

Hydrogen bonding accounts for many of the singular properties of water, such as its high boiling point, it high surface tension, its wetting and solution properties, etc. Its density/temperature relationship is among them. Water is one of few substance (diamonds are another), all of which are in the tetrahedral crystalline shape, whose molecular arrangements in the solid, crystalline state (ice) are less dense than they are in the liquid state. As a result, ice floats on water, with enormous effects upon terrestrial life. The hydrogen bond is a weak bond, on the order of 4.5 kcal/mol as compared to a covalent bond at 110 kcal/mol for an O–H bond. However, it is an extremely important bond. It has a prominence in protein chemistry, having strong influences in the associative bonding common to proteins, and it plays a major role in maintaining the structural integrity of many biological macromolecules.

The hydrogen bond, represented formally by three dashes, is weak enough to be broken easily by the molecular mobility of the liquid state, only to be reformed immediately with new neighboring water molecules. Facile though the bond may be, it represents a definite force. Thus, water molecules being electromagnetically associated, are not gaseous at room temperature as their molecular weight of 18 would suggest, but form a rather high-boiling liquid (100˚C).

Solvating Effects

An example of the water molecule's wetting and solvating capability follows: The electrolytic salt molecules considered above are ions that exist as molecular entities as long as the electrical ionic bond created by the attractions of opposite charges persists. The ionic bond can, however, be weakened and disrupted by the insinuation of electrically charged structures between the sodium and chlorine moieties, thus attenuating their strong mutual attraction. The addition of water to an electrolyte, such as salt, affects this ion/dipole interaction. Water, because of the electronegativity of its oxygen atom and the electropositivity of its hydrogens, is a dipolar molecule with a high dielectric charge; its oxygen atom has a partial, hence weak, negative charge, and its two hydrogen atoms have each a partial positive charge (Tanford, 1980, Chap. 5).

The water molecules, by way of their partial charges, respond to the electrical charge forces of the ions, causing them to become hydrated. That is, the ions acquire skirts of water molecules attached by the electrical attractions of opposite charges. These new electrical alliances compete with and dilute and weaken the power of the primary ionic bonds forming the salt molecule. Heretofore, the electrical needs of the ionic charges had been exclusively satisfied by the counterions, but these interactions are now compromised by the competing dipolar influences of the many water molecules. The water separates the ionic lattices by displacing the ionic bonds with its own dipolar alliances. This brings the salt into solution. That is to say, each ion is now individual, released from its ionic lattice, and separated from the others by an envelope of water molecules that are attached to one another within their hydrogen bonded structures. The ion size is increased by its hydration. This can have implications where the ion-size is a consideration. The charge density of the hydrated ion is less than that of the ion itself; the mass over which the charge is spread being greater. Organic compounds can also acquire waters of hydration if they contain partial charges that interact with their opposites on the water molecules.

An interesting view of the adsorptive bond formation that ultimately attaches the particle to the filter involves a competition between two forces. Hydrophilic sites on surfaces become wetted because of their strong mutual attraction with water molecules. In order for two such wet solid-surfaces to interact with one another, the water molecules bonded to each of them must be displaced. This dislodgment can only be effected by stronger surface-to-surface forces.

van der Waals Forces

There are charge/charge (ion/ion) interactions; ion or charge/dipole, and dipole/dipole charge interactions. There are also charge/induced-dipoles, and dipole/induced dipoles. Overall, the strength of the bonding is greatest where charges are involved; is less for permanent dipoles; and is least for induced dipoles. The strength of the bonding depends upon the asymmetry of the molecule's charge distribution, the distance of separation, and the value of the dielectric constant (Gabler, 1978, Chap. 5). In all these cases some molecular polar entity can be recognized as being the originating cause. However, a similar imbalance of electrons may come about in molecules where no polar influence is evident. These are ascribed to induced-dipole/induced-dipole electrostatic forces. They are the London–van der Waals dispersion forces, named for their early investigators. They serve as a weak but very important attraction mechanism. They were deduced from experimental investigations of departures from

the Perfect Gas Law. The noble gases[b] are inert because their outer electron orbitals are completely filled. Therefore, they do not form covalent bonds, not being in need of electron donating or borrowing. It was found, nevertheless, that they exhibit electronic attractions. This, it is theorized, is the result of partial charge interactions not involving valence bond formation.

As a consequence of their obscurity, but with an appreciation of their reality, there are widespread mistaken references in the technical literature to the VDW forces as regards their genesis. All attractive forces of partial charge origins are often referred to as VDW forces or as "secondary valence" effects. Albeit incorrectly designated, the end result serves in that there is recognition that unsatisfied electronic expressions are at work. The VDW forces are universally operative, but are seen to be of prime importance among non-polar molecules, such as hydrocarbons, whose non-polar structures would seem not to hold possibilities for inducing dipole formation.

The VDW forces are fundamentally different from the classical models of the electrical interactions just considered. The VDW attractions are ascribed to *transient dipoles* that result from an "instantaneous non-zero dipole moment" that induces a momentary dipole in a neighboring molecule. (Gabler, 1978, Chap. 6) Electrons are in constant circulation around their nucleus. Therefore, the charge distribution, over a time period, is not in one fixed position. It is described in terms of a "cloud" to emphasize its ubiquitous positioning. However, although constantly changing, the molecule does at any instance have an immediate dipole moment. It is this that induces dipoles in adjacent molecules. The VDW forces operate as attractive influences, albeit weak and effective over only short distances. However, in their multitude they are of substantial import. A molecule is not limited to a single fluctuating dipole, but may have many transient dipoles, each capable of inducing a dipole in another molecule. The VDW, therefore, has a cumulative effect (Gabler, 1978, Chap. 6).

It may be of interest to know that in addition to being responsible for the adsorption of organisms and other particles to filter surfaces, and its similar action in destabilizing colloids, VDW forces govern the condensation of gases into liquids by their induced-dipole, induced-dipole interaction. For instance, in the gaseous state, water molecules, like all vapors, are widely separated and remain so. When, however, they are squeezed together by pump action, the attractive forces acting among them become operative over the compressed intermolecular distances, and overcome the ever-present repulsive forces. Liquid water is thus created. (This is the operative principle of the vapor compression still whose external pressure brings together the water-vapor molecules and coalesces them into the greater intimacy of the liquid state.) The closer molecular proximity of the liquid state accounts for its higher density. The dipole/dipole attractive force resulting from the thermal condensation of steam has the same effect, as does also the induced VDW. Because of its effect, the VDW has been described as being an internal pressure. VDW forces are involved in lipid-lipid interactions, in interactions among hydrocarbons, and even, as stated, among the noble gases.

Bonds of Partial Charges

Obviously, there are numerous bond configurations that result from the partial sharing of the covalent electrons. These differ in their origins and strengths. Not all are significant to the desired particle retentions by filters. The presently accepted theory accounting for a

[b] The noble gases are Helium, Argon, Neon, Krypton, and Radon.

filter's ability to remove particles focuses upon those interactions that are governing. In addition to the ionic bonding there is a variety of partial charge interactions: charge–charge arrangements; charge–dipole combinations; dipole–dipole reactions; charge-induced dipole interactions; and induced dipole–induced dipole dispositions (Gabler, 1978). Three of these bond types in addition to the ionic types are generally considered to be the influences that in their interplay of repulsions and attractions achieve the balance necessary for particle retentions. These interactions differ in their strengths and in the distances over which they exercise their powers.

Of the two interactions that exhibit the repulsive behavior of like-charged entities, the more powerful is the mutual reaction of full ionic charges. Coulomb's Law describes the situation. It holds that the electrical force between two poles is the direct result of the difference in the charge between them, and that its action is direct and strong enough to operate over long distances. The law established that like-charges repel, and unlike-charges attract. In the case of organism and filter, both acquire the same charge designations from the solution that surrounds them. Its influence decays inversely as the square of the distance between the poles. Another repulsive force is the more general repulsion exercised when any two atoms are brought together close enough for their (negatively charged) electron clouds to overlap. These forces are limited to the short distances at which they are generated. The mutual repulsions of the like-charges of the strong primary (ionic) forces that are operative over the longer distances are dominant.

The interactive forces that project an attraction between organism and filter surfaces are seen to be the result of the mutual responses of unlike-charged atoms or molecules. These are generally ascribed to forces arising from quantum mechanical origins; from electrostatic interactions; and from VDW forces.

The distance separating the two surfaces is of prime importance. The long range coulombic mutual rejections overwhelm the attractive short range powers. At low ionic strengths the attractive influences become dominant only at a very short distance of separation between the two surfaces. The separation distance is termed the Debye length. The greater the Debye length as caused by a sizable (zeta) potential, the greater the influence of the mutual repulsion involving like-charged surfaces. As will be discussed, the Debye lengths can be shortened to the point where the attractive forces compel the surface-to-surface bonding of particle and filter.

To summarize: the attractive forces are short-range, whether electrostatic, quantum mechanical, etc. They are the products of interactions, that include the VDW forces. When the two surfaces are at a distance, the repulsive forces, being stronger, dominate. With decreasing separation distances the attractive impulses increase relative to those that repel. When the distance separating the two surfaces is reduced sufficiently, the attractive forces can assert themselves. At this point the adsorption of particles to filters, or other solid-surface interactions occur. Adamson (1982, p. 232) states that at a separation distance of a particle's diameter the attractive forces are at a maximum. Beyond this point repulsions again set in. The cause of this repulsion is the overlapping of the electron clouds. It is of a very short range, and manifests itself rapidly as atoms or molecules come within a certain distance of one another.

Quantum-Mechanical Forces

Although the valence needs are satisfied by the union of two atoms, the molecule thus formed may still attract others around it due to the partial charges just discussed. These residual weaker forces have a strength of about 0.5–5 kcal. This force level, associated

with a two-atom molecule, is too small to effect bonding. However, these forces are additive. Where the molecule is composed of many atoms, as in the case of polymers, the residual force can be considerable. It is then conducive to bonding. As Zydney (1996, p. 399), describes, it is these quantum-mechanical forces that operate at short ranges to attract affected surfaces to one another. This leads to electron sharing and bonding between the partnering atoms. This type of interaction exerts an attractive force helpful in particle removals. Electrostatic attractions and the VDW forces are two other phenomena that exercise attractions between surfaces.

There is, however, a potential and practical downside to this interaction. It can occur between macromolecular species present in the solution leading them to aggregate into larger entities that may contribute to filter fouling. Be that as it may, such positive quantum-mechanical interactions are among the attractive forces that adhere particles to filters.

Electrostatic Interactions

Aside from the electrical charges that derive from their molecular structures, surfaces of macromolecular units such as filters, organisms and other large elements acquire fixed charges from their exposure to aqueous solutions that almost certainly containing ions. Negative ions seem to undergo preferential adsorptions onto surfaces especially at neutral pHs. At alkaline pHs anion (negative) adsorptions predominate. Negative surface charges may also result from the ionic dissociation of carboxylic acids created by the oxidation of organic molecules.

Electrically charged surface-atoms, whether of ionic or covalent origins generate electrostatic responses in the atoms of adjacent molecules. Mutual attractions or repulsions result respectively from unlike or like-charged neighbors. Thus, interactions arise, as stated, from the fixed charges of opposite signs and from fixed and charge-induced dipoles (Gabler, 1978, p. 180). The negative charges adsorbed and fixed to the filter, and/or other particle surfaces attract and firmly bind a layer of counterions. The formation of a layer of ions of opposite charge is in accord with the Debye-Hückel theory. This layered combination of fixedly adsorbed ions and counterions forms the first of the electric double layer arrangement that is central to the adsorptive bonding phenomenon (Fig. 24). The attractive forces that are ultimately responsible for the

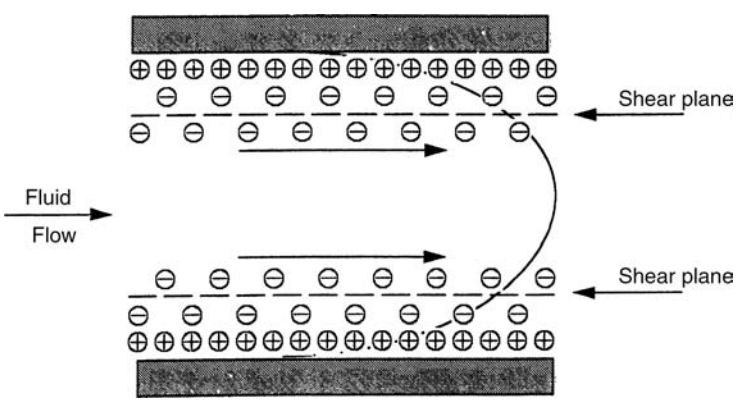


FIGURE 24 Ion distribution near the wall of a positively charged capillary. *Source*: From Schmidt and Marlies, 1948.

bonding of particle surfaces to filter surfaces derive from such electrostatic interactions. The accepted accounting for the adsorptive interaction of surfaces, shortly to be discussed, is named the DLVO theory. It can be considered an extension or an elaboration of the Debye–Hückel theory. Adamson (1982, p. 215) depicts a detailed model of the double layer (Fig. 25).

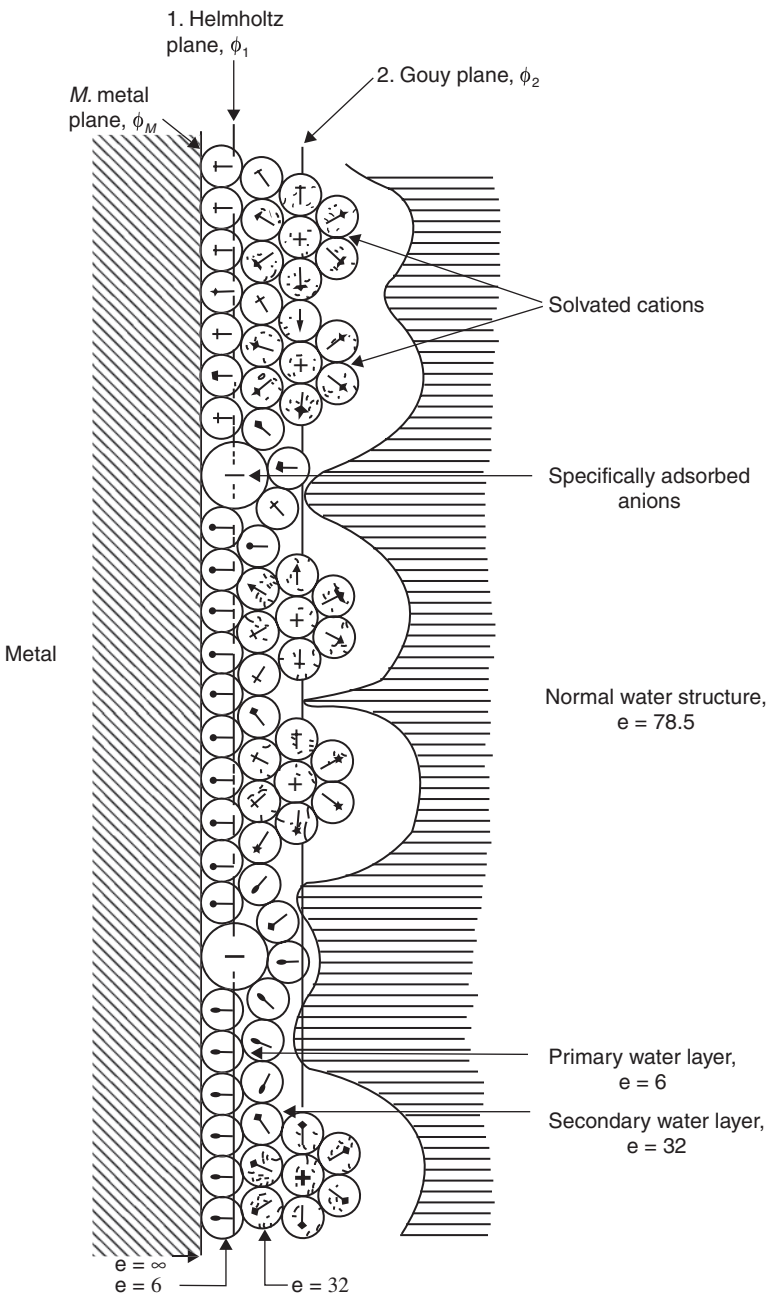


FIGURE 25 A detailed model of the double layer. *Source*: From Adamson, 1982.

Free Surface Energy

As a result of the surface interaction between filter and particle there is a reduction in the system's free surface energy. The result is a more stable molecular arrangement. A proper treatment of the subject of free surface energy requires the application of thermodynamics. A less ambitious effort is being made here to set forth the concept of free surface energy in a less rigorous manner. The beneficiaries will be those who, like the authors, are limited in their ability to probe the occult and mysterious.

The situation being considered is that of a filter and of an organism both immersed in an aqueous solution. All molecules have a certain energy available for interactively relating to their surrounding, neighboring molecules provided proper conditions exist. Common to all surfaces, their molecules differ from those within the mass because they interface with a surrounding gas phase, namely, the air. Being a gas, air has a low density. It is sparse in its molecular population. The surface layer of the solid object is, thus, molecularly unsaturated. The molecules composing it possess a residual field that is high in Gibbs' free surface energy.

Consider a droplet of water. Every water molecule within its depth will interact with its neighbors to the full extent of its powers to undergo bonding. As a result, a given volume of water will, in effect, consist of a network of water molecules connected to one another by hydrogen bonds (see the section on "Hydrogen Bonding"). The propensity of the water molecules to H-bond is a measure of the energy available to them for interaction with other (suitable type) molecules.

However, the water molecules situated on the spherical boundaries of the water droplet and constituting its interface with non-water molecules have few neighbors on the droplet's periphery suitable for bonding. The unexpended energy remaining in their non-bonded region is the free surface energy being discussed. When a liquid is dispersed into discrete multi-droplets the mass of water assumes a proportionally larger surface area; and, therefore, a larger total free surface energy. The free surface energy of the multi-droplets expended in bonding interactions serves to coalesce them into larger volumes with a consequent diminishment in the liquid's total surface area. A loss results in the total free surface energy. This is a consequence of the adsorptive interaction.

ATTRACTIVE AND REPULSIVE FORCES

It may be advantageous to again address the balancing of the attractive and repulsive forces whose management can culminate in particle removals by filtration; especially so for those of us for whom the "electric double layer" is a relatively new concept. The electric double layer governs the adsorption of organisms to filter surfaces. It forms simultaneously on the surfaces of both the filter and the particle when they are exposed to an aqueous medium. Being like-charged, the two surfaces exhibit a mutual repellency.

In a filtration both the particle and filter are positioned within the aqueous stream. As is inevitable, both surfaces, because of their higher energies, acquire ions from the solution, or gain charges induced thereby. The acquired ions become firmly fixed to the immersed surfaces. In effect, the surfaces of both filter and organism simultaneously accumulate electrical forces of both stronger and weaker powers, and of both attractive and repulsive capabilities. The magnitude of these effects is proportional to the charge density on the individual adsorbing surface. The acquired ions give rise to strong and, hence, long range coulombic forces. In the case of organism and filter, both acquire the same charge designations from the solution. Therefore, the coulombic forces are mutually

and strongly repellant and are exercised over long distances. The concurrent attracting forces are short range, e.g., van der Waals, and others of partial-charge origins. They are too weak to redress the repulsion. That they are effective only at short distances is a measure of their weaker influences.

As would be expected, the electric potential of the adsorptively-fixed ionic surface charges attract counterions, ions of opposite charge from within the solution that become fixedly attached to them. These, being electric conductors, effectively shield and moderate the electrically repellant interactions between the two charged surfaces, namely, of filter and of organism. In consequence, the electrostatic forces are reduced in potential. However, not all of the fixed surface charges are neutralized or shielded by the inseparably-attached counterions. Consequently, additional ions of opposite charge, intermingled with *their counterions,* are attracted by the fixed surface-charges that remain un-neutralized. However, these augmenting counterions remain mobile within the solution because, although responsive to the fixed surface charges, they are increasingly distant from them, and the interactive forces diminish sharply with distance. The mobile ions, by definition, can be caused to migrate by the impress of an electric current.

The fixed surface charges and their *firmly held* countersigned ions constitute the first layer of the electric double layer. The second layer is composed of the *mobile* counterions. The double layer potential extends from the line of counterion separation as far into the liquid as is necessary in its search to include enough countersigned ions to satiate the remaining unsatisfied fixed charges on the solid's surface. The electrical force generating the attraction of the mobile counterions is called the zeta potential. The distance it extends into the solution in its seeking after counterions is known as the Debye length, (symbol K^{-1}).

The zeta potential will shortly be treated in detail. Postponing its consideration permits a less disjointed account of how the electric double layer functions.

Attenuation of Repulsive Forces

It is evident from the foregoing that empowering the attractive VDW forces requires the shortening of the Debye length. This can be accomplished by the addition of ions in the form of salts. Contrarily, the low ionic content of dilute solutions results in extending the Debye lengths. This renders the weak attractive forces impotent against the strong long range coulombic forces of repulsion. The addition of ions to the solution makes possible the dominance of the attractive forces. The ionic strength of a solution is a measure of its salt or ion content. Upon the reduction of the Debye length as occasioned by the addition of ions, the short range attractive VDW forces enable the appropriate surface sites on the organism to interact with those on the filter. Since there are as many opportunities for the VDW force orientations to exercise repulsions, as there are to encourage attractions, the domination by the attractive tendencies requires explanation. Actually, both the attractive and repulsive forces are reduced by the addition of ions, but the repulsive power is the one more affected. On balance the attractive forces emerge the stronger.

It turns out that two factors incline in favor of the attractive forces. First, when placed in an electric field the induced dipoles that are the VDW forces can align themselves either in a head-to-head and tail-to-tail, or head-to-tail sequence. This reflects a probability factor. The head-to-tail arrangement, alternating the positive and negative partial-charge interactions involves a lower energy level and thereby signals its greater stability. The greater stability, therefore, favors this orientation, and it is this orientation

that more effectively shields and neutralizes the zeta potential. Instituting a chain of subsidiary or partial charge interactions, in the form of an array of dipoles, between two like-charged repulsive forces such as reside on particle and filter, serves to attenuate the mutual antagonistic, repellent interactions.

This is abetted by a polarization factor. The VDW force, also called the London dispersion force, affects the size of the dipole moment by influencing the spatial separation of the plus and minus charges. These exist in each molecule in proportion to the dipole sizes. In the electrostatic attraction mode, polarization increases the interaction between the two molecules. In the repulsive orientation, the polarization decreases the molecular moments; hence, the repulsive force. As stated by Wheland (1947), "the attractive orientations on the average, are more attractive than the repulsive orientations are repulsive." As a result of these two factors, the short range VDW forces exert an attractive influence, and bring about the desired filtrative removal of organisms by an adsorptive interaction.

Incidentally, the equilibrium point where the attractive and repulsive forces balance one another defines the space that exists between the atoms composing molecules, and also between the overlapping segments of long polymeric chains.

Perhaps the influence of ionic strength can be made more apparent by focusing on the effects of the "ion cloud" created by the addition of salts. "The counterions screen the central molecule and reduce the strength of its net charge" according to Gabler (1978, Chap. 7); also, "the (electron) cloud shrinks about the macromolecules making the entire unit more compact." Its size is reduced. The ions of the opposite polarity tend to cluster closer to the macromolecule. In so doing they dilute or shield its effective charge. The crowding grows proportionately closer as the concentration of counter-charged ions increases. A more effective screening of the charge takes place. This compression enables the charged macromolecules to approach each other closely enough to overcome the repulsive forces.

The exemption of the attractive forces from the shielding powers of the electron cloud is more apparent than real. The attractive forces are also reduced. However, "the counterion cloud dilutes the net charge on the macromolecule more so than it does the VDW forces." The attractive VDW forces created by the dipole oscillation from one orientation to another are, unlike the net charges, not disrupted because the counterion distribution cannot orient fast enough to conform to the dipole oscillations.

Gabler states, "The key to the whole explanation is the fact that as the salt concentration increases, the Debye length, which measures the radius of the shell of counterions about the macromolecule, decreases." As a result of the greater diminishment of the repulsive forces, the countervailing forces of attraction assert themselves, and the two surfaces attain interaction by way of the adsorptive sequestration mechanism.

It is this effect that minimizes the strength of the (repelling) zeta potential. This, then, describes the working of the electric double layer in furthering the filtration operation in its adsorptive removal of particles from suspensions.

Debye-Length Associated Phenomena

Fletcher (1996) describes the attractive and repulsive forces involved in the interactions between organisms and the filter or other surfaces, and offers quantification of the approximate distances at which they are significant: At distances greater than 50 nm, called the "secondary minimum," VDW attractive forces do cause the positioning of the

bacteria nearer the surface; but so weakly that they are readily removed by shear forces. At distances of 10–20 nm, repulsive electrostatic forces still dominate. Here the Debye length is too great for the weaker VDW attractive forces to prevail. At between 2 and 10 nm both repulsive and electrostatic attractive forces are manifest, the distance is now small enough to allow the weaker attractive forces to begin asserting themselves. At between 0.5 and 2 nm, the water adhering to the surfaces is still a barrier to specific surface interactions. However, if the two surfaces include non-polar areas or patches, these may coalesce to form hydrophobic adsorptions concomitant with the elimination of the intervening water. At less than 1.0 nm, the attractive forces are strong enough to cause specific organism-surface interactions to take place (Fig. 26).

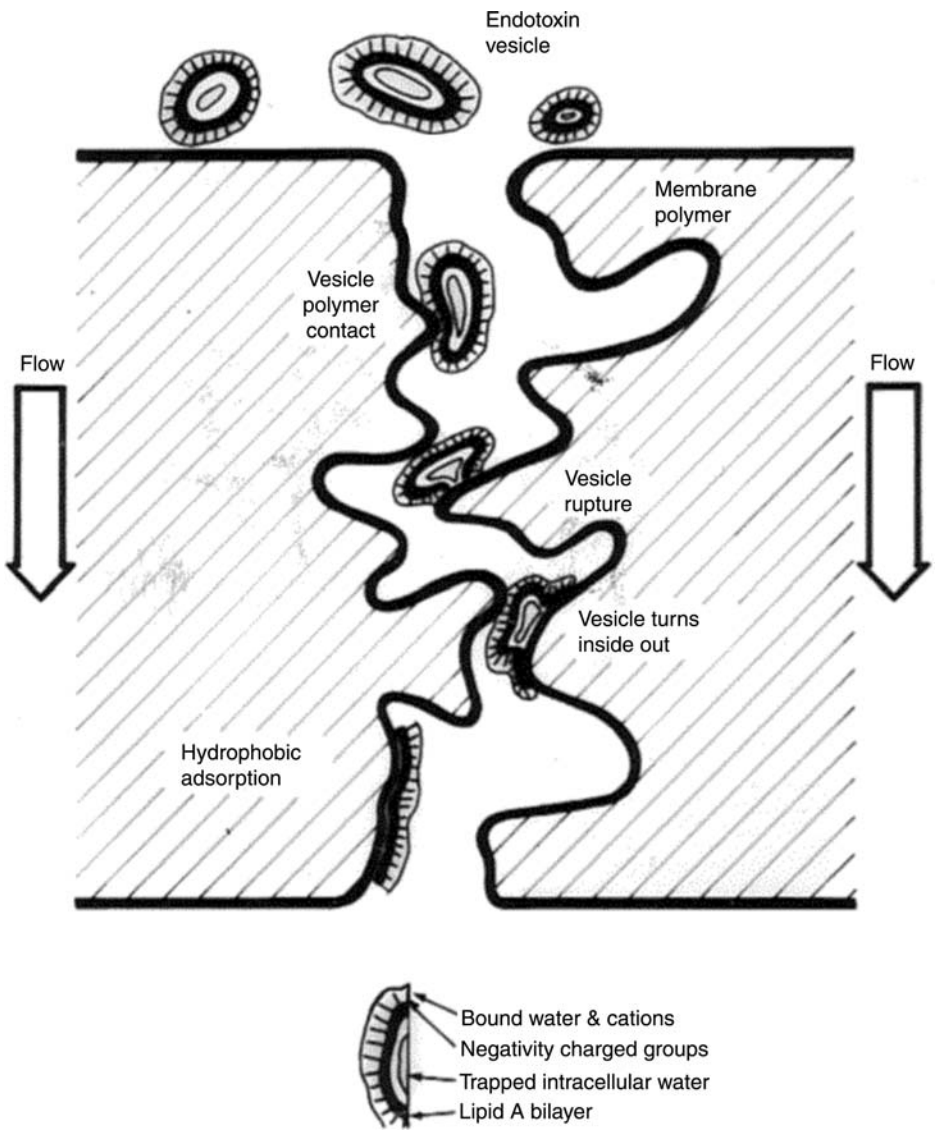


FIGURE 26 Hydrophobic adsorption mechanism. *Source*: From Robinson et al., 1982.

In agreement with this observation, Pall et al. (1980) state that when the Debye length corresponds to a zeta potential of less than 30 mV, the VDW forces are able to effect the adsorptive interaction. Colloids are stabilized when their particles are endowed with net surface charges of similar sign in the magnitude of 30–40 mV or more.

There are, however, limitations to attaining the required small Debye lengths. As mentioned, the adsorption of a surfactant molecule onto a macromolecule's surface increases its size. This steric hindrance may keep it too far from the other surface to yield a sufficiently short Debye length. Non-ionic surfactants in particular contribute to such a condition. The two surfaces are, thus, sterically stabilized against adsorptive coalescence. Spatial interferences can also result from the hydration barriers created by the increase in an ion's size by its acquisition of waters of hydration. Such steric interferences can create an energy barrier sufficient to frustrate adsorptions.

Bacterial Adhesion to Surfaces

It would seem that the adsorption of organisms from their suspensions onto solid surfaces would involves the same forces of attraction and repulsion that regulate the adsorptive joining of one colloidal particle's surface to that of another, as just discussed. The process involving organisms would explain their retention by filters. It would also seem to be central to the establishment of biofilms, concentrations of microbes, dead and alive, nutrients and their metabolic products. Organisms seem compelled to seek attachment to surfaces. It acts as a survival mechanism. As attachments to surfaces, organisms secure protection from sterilants such as active chlorine preparations. The extracellular polymeric substances (EPS) within which the organisms are embedded in the biofilm serve to shield them from antagonistic agents, including a free chlorine residual of several ppm (Le Chevallier et al., 1984; Ridgway and Olson, 1982; Seyfried and Fraser, 1989).

Marshall (1992) points out that in conformity with accepted theory, bacteria, as also particles in general, are kept from contact with surfaces by electrostatic forces. This includes the surfaces of other organisms. The repulsive forces involved, as discussed, are effective at long range, and prevail over the weaker attractive forces that are simultaneously present. As described in the discussion of the electric double layer, the repulsions are attenuated by higher electrolyte concentrations. The attractive forces, albeit weaker, can then assert their influence. The adsorptive interactions that follow result in the formation of a biofilm.

However, according to Marshall, the repulsive forces may still be strong enough to keep relatively larger bacteria from directly contacting the surface to which they are attracted. Steric hindrance, a result of the organism's large size, would be the cause. Were this the case in the matter of forming biofilm, some authorities believe that the too large separation distances could be bridged by an extracellular slime (EPS) exuded by the organisms. The EPS is constituted of complex molecular structures such as heteropolysaccharides, and glycoproteins. Presumably, these exudates overarch the too large separation distances to establish the biofilm. Nevertheless, there is also the opposite view, namely, that the attachments are other than a result of such metabolic processes as the exopolymer-mediated bridging. In this view, the adsorptive attachments are exactly those resulting from the electric double layer phenomenon. In this manner, it is believed, the organisms become attached to the filter surface, and to one another. Biofilm development is the result.

The Electrical Double Layer

The formation of the electrical double layer and its consequences in terms of charge distributions and the distances over which they hold sway are basic to the understanding of the adsorption mechanism. To recapitulate in the interest of clarity, all surfaces, whether of filters or organisms, etc, acquire a net surface-charge of fixed ions when immersed in an aqueous solution (certain to contain ions). This may eventuate from the adsorption of specific ion from the solution, or from the ionic dissociation of functional groups that are part of the surface's molecular structure. According to Zydney (1996) at neutral pH most microporous membrane surfaces preferentially adsorb negative ions, such as carboxylic acids, and become negatively charged. The carboxylic acid group on a surface is likely to result from the almost inevitable time dependent oxidation of organic molecules that form parts of the particle or filter surface. Alternatively, positive charges may become adsorbed to become a permanent part of the filter surface.

Regardless of the origin or sign of the charge, its presence endows the surface with an electrical potential that is a function of the charge density. These surface charges will alter the arrangement of the ion concentration within the solution by attracting ions of opposite charge. Such counterions will form a fixedly joined layer to the acquired surface charges already bonded in an inseparable union. This will, by dint of its opposite charge, attenuate the original electrical potential of the surface charges. The result is a single but biphasic overlay of oppositely charged sites on the surface. However, the attracted and fixedly held counterions within this overlay are deficient in number to the surface charges that attract them.

The negative charges on the surface remaining unshielded by counterions represent the net electric potential of this biphasic layer. The as yet unappeased surface charge extends its influence into the liquid for additional positive ions to more completely fulfill its charge needs; and visa versa if positively charged. This potential will project into the solution to a distance known as the Debye length attracting additional counterions within the solution. The counterions, the electrical load carriers, will form successive charge-neutralizing zones throughout the liquid; more diffused but with increasingly less charge-homogeneity as the charge attractions progressively attenuate with distance.

The counterions fixed to the surface charges are immobilized. They are firmly bonded, whether to the organism or filter surface. However, the attracted counterions more distant within the solution become increasingly less firmly bonded according to their distance from the fixed surface charges. Within a certain zone, there is a line, (more properly a band), of separation between the fixed counterions and those that could exhibit mobility in response to an imposed direct electric current. Were such to be applied, a line or zone of separation would become manifest between the mobile and immobile counterions. The zeta potential extends within the solution from this line of separation in its search for ions of opposite charge to satisfy its un-assuaged fixed charges. The more dilute the solution, i.e., the fewer the ions, the larger the Debye length.

It may be useful to consider the disposition of the charges as being in the form of a capacitor. Gabler (1978, Chap. 3) provides a necessary source. The subject arrangement consisting of two connected plates oppositely charged by an electrical source, but separated by a vacuum extensive enough to insulate one plate from the other. Consider the introduction of a dielectric between the plates. Although the dielectric molecule remains electrically neutral, opposite charges build on its surfaces next to the plates. The dipoles, originally randomly arranged, align themselves so that their electrical fields oppose the field causing the orientation. This polarization of the dielectric structures neutralizes or dilutes the charges on the plates. The upshot is a reduction in the voltage or

electric field between them. The field of a charge reflects its surrounding medium. Therefore, the presence of water, or any other dielectric, reduces a charge's electrical field to a level less than it would exhibit in a vacuum. It is this moderation of the zeta potential that enables the diminution of the Debye length to the point where adsorptive bonding between surfaces can take place.

The Zeta Potential

The first part of the electrical double layer consists, as said, of the charges sited on the boundary surface of the solid (filter), and the (hydrated) counterions that are permanently attracted and bound to it. It is called the Stern layer or Helmholtz layer. The second layer, measured from the line of separation, consists of the diffuse, less strongly bound layer of less homogeneous ions assembled within the solution in response to the zeta potential. It is called the Gouy or Gouy-Chapman layer (Fig. 27). The application of an electric current upon this arrangement causes the migration within the Gouy layer of the less firmly fixed counterions ions it contains. A line of shear will form, as stated, between the fixed counterions, and those present and migrating in the liquid bulk. The Debye length measures the distance over which the zeta potential extends within the solution to satisfy its need for ions of opposite sign.

The same phenomenon manifests itself on both the filter and organism surfaces. The larger the zeta potential as caused by the paucity of fixedly attached counterions, the greater the Debye length separating the two surfaces, and the stronger their mutual repulsion.

The net effect of the double layers is the development of the zeta potential. Of similar sign on both the filter and organism surfaces, its repellant force inhibits the mutual approach of the particle surfaces to that of the filter. In the case of colloids it prevents the approach of one colloidal particle to another. This preserves the stability of colloidal suspensions, and countervails organism adsorption to filters. The capacities for attractive and repulsive forces between any two surfaces are simultaneously present. The attractive forces can act only over smaller distances because they are relatively weak. Their more limited powers reflect their origins as being derived from partial-charges;

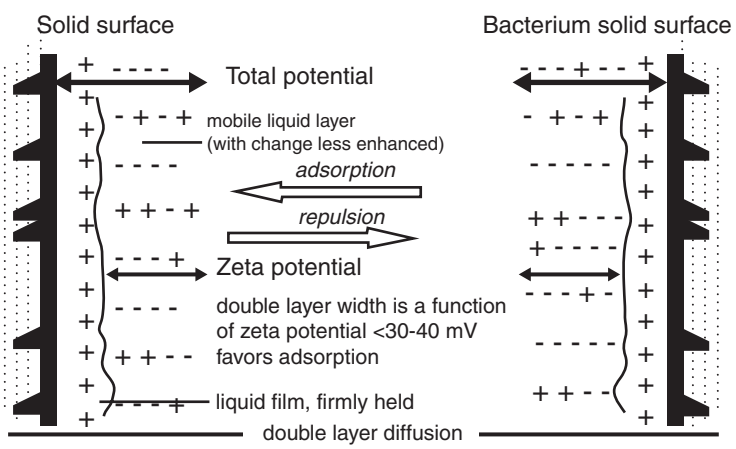


FIGURE 27 Electric double layer. *Source*: From Mittleman et al., 1998.

namely VDW forces, and hydrophobic adsorptions. At moderate to large distances, the electrical potential arising from the partial charges decay exponentially in an electrolyte solution with a characteristic length scale equal to the thickness of the double layer (Zydney, 1996). They operate at the twelfth power of the distance. Thus, these electrostatic attractive forces become negligible when the surfaces are separated by more than a few Debye lengths. However, the repulsive forces are stronger. They mirror the stronger influence of full charges.

The interpositioning of ions between the two repelling charges attenuates their mutual repulsion. Thus, the zeta potential, can be moderated by managing the ionic strength of the solution, as by the addition of electrolytes. The ions from the dissolved electrolytes provide an electrostatic shielding of the zeta potential, causing its diminishment, and the shortening of the Debye length. The magnitude of the electrostatic interactions between the charges on the membrane surface and the ions of the second layer is, therefore, a strong function of the ionic strength of the solution. The zeta potential reduction by the addition of ions is the customary practice in water treatments utilizing coagulation with alum to settle colloids. The zeta charge-moderating ions being made available, a shorter Debye length obtains. The diminishment of the double layer distance, by the addition of Al^{+3} ions, promotes the colloid destabilization and agglomeration that is the water purification's objective. The adsorptive sequestration of particles to filter surfaces results from a like-effect enabled by solutions of high ionic strengths or of high osmolarities to moderate the long range repulsive forces and to permit the short range VDW attractive forces to dominate.

Restatement and Elaboration

The measure of the zeta potential represented by the symbol ζ is the difference in the electrokinetic potential that exists within the Gouy layer over the entire mobile counterion expanse. It is a measure of the charge on the filter surface that is not satisfied by the permanently bound counterions (Figs. 24 and 27). The counter charges required for electrical balance must, therefore, come from the mobile, less tightly bound ions within the liquid phase. Commencing at the line of shear, the zeta potential extends within the bulk of the solution encompassing the mobile counterions it influences.

The thickness of the double layer relative to the particle diameter is very small. In a 10.3 molar monovalent ion solution the thickness is approximately 100 Å or 0.01 μm (American Water Works Association, 1969). Nevertheless, a reduction to such a Debye length is needed as it enables the attractive forces to take over. Its magnitude is inversely related to the ease of destabilizing colloid particles, and/or encouraging adsorptions. Both are exercises that involve double layer shrinkage and charge neutralization. The lower the zeta potential, the smaller the distance, the Debye length, between the permanent charges of the first surface, and the fixed, opposite charges of the second surface. Adsorption follows from the interaction of the two (opposite) charges. The precise location of the shear plane, the Stern layer, is not known with exactness since it depends on the adsorbed ions and their degrees of hydration. Nevertheless, zeta potential measurements are useful in characterizing the surfaces of particles, including bacteria. Its values relate to the coagulation-flocculation process, to colloid destabilizations, but only when charge-neutralization is the only consideration (Bratby, 1980, Sec. 2.8). This is not always the case, and its predictions may differ from the classical jar-test results (Bratby, 1980, Sec. 8.1). There are, then, limitations to zeta potential predictions of performance.

According to Cohn's Rule, a particle suspended in water acquires a zeta potential that reflects both its and the water's dielectric constant (Johnston, 2004, Chap. 9.2). Water has a dielectric constant of 72. If the particle has a higher dielectric value, its zeta potential in water is positive. (Asbestos is a case in point.) If the dielectric charge of the particle is lower, its zeta potential is negative. Most particles suspended in water bear negative charges.

An intriguing view of the effects of the zeta potential, the measure of the electrokinetic effect, *vis a vis* colloids is given by Pall et al. (1980). These investigators point out that colloidal suspensions are stabilized when their particles are endowed with net surface charges of similar sign in the magnitude of 30-40 mV or more. The mutually repulsive forces then suffice to repel the particles from one another. The double layer distance is then large enough to frustrate the shorter range attractive VDW forces. Therefore, no flocculation occurs, and the colloidal dispersion is stabilized. Below about 30 mV the double layer extent shortens, and the zeta potentials begin to reflect the growing involvement of the attractive secondary valence forces. Marshall (1992) considers the critical Debye length to be from 10 to 20 nm, at which point "... long range (sic) van der Waal attractive forces can exceed 'the' electrical repulsion forces ...." Over and at the zero charge level, attraction dominates and flocculation occurs: The colloid becomes destabilized.

To be sure, the ionic strength of the suspending solution, like its pH, exerts a restraining influence on the magnitude of the mutually repelling like-charges. Low pH (high hydronium ion, H_2O–H+), and/or high ion concentrations serve to attenuate the repulsive forces. They shorten the Debye length, and so usually promote flocculation in colloids. They also tend to influence adsorptive particle arrests. Nonionic surfactants also exert an influence. The particles are enlarged in size by the hydrophobic adsorption of an enveloping nonionic surfactant coating. The increase in their size abbreviates the charge density which now becomes dispersed over a larger area. This shortens the Debye length, making it possible for the attractive forces to come into play. The separating effect of the repulsive potential energy barrier that prevented the attractive oppositely charged particles from approaching one another is overcome at the shortened distances. The phenomenon is referred to as steric stabilization. It has also been called entropic stabilization. The spatial barrier effect, or Debye length may be of a considerable magnitude. Nonionic surfactants in particular can exert a significant influence on overcoming its colloid stabilization effect; likewise on the adsorptive sequestration of particles by filters.

Jaisinghani and Verdigan (1982) provide a discussion of how to measure the zeta potential of a filter medium. However, the measurements of zeta potential, even by the zeta meters devised for that purpose, are time-consuming to a degree that reduces their practicality in assaying, for example, the quantity of alum needed for the clarification of a water to rid it, by agglomeration, of its colloidal content. The time-honored jar tests are often used for this purpose. Their results are considered more dependable (Bratby, 1980, Sec. 8.1). As stated, there are limitations to the reliability of predictions of performance based on zeta potential. Some consider zeta meters as less than perfect instruments, and view their use as troublesome (Mandaro, 1987, pp. 187–93). They prefer to measure the streaming current potential.

Streaming Current Potential

In the measurement of zeta potential, the core particle with its attached charges is caused to separate from its charge envelope by being moved electrically through the suspending

(non-flowing) water toward an electrode. The same separation of the electrical double layers can be obtained by anchoring the particles, as by adsorption to surfaces, and causing the liquid to flow past them. This is called the streaming current potential technique.

It is easier to perform than zeta potential measurements, and it too measures the voltage necessary to separate the double layers and, hence, helps determine the ease of colloid destabilization or particle adsorption. It does so by providing a measurement of the net surface charge of the colloidal particles. This correlates with how much coagulant must be added to the colloidal suspension to cause it to agglomerate. The coagulant, such as alum, supplies multivalent cations, Al^{+3}, to neutralize the negative charges of the first electrical layer. This charge neutralization destabilizes the colloidal suspension, *reducing the double layer dimension,* the Debye length, and thus permitting the particles to agglomerate and to become large enough to be responsive to gravitational settling.

Streaming current measurements are more speedily performed, and the results are less subjective. They offer the advantage of being immediately expressed as the average for the system. This technique reads an alternating current on a direct current meter. The polarity of the output is adjusted to indicate the surface charge. Streaming current measurements are relative, and are dependent on the very streaming current detector used. In general however, their values correspond to the optimum destabilization points given by jar tests using particular coagulants.

Knight and Ostreicher (1981) described a suitable instrument, and reported on the use streaming-current potential measurements. In potable water treatment plants, such devices are relied upon to assess the coagulation requirements of water containing colloids, i.e., to gauge the amount of alum needed to destabilize the colloids. The findings are, however, usually confirmed by coagulation jar-tests. Knight and Oestreicher (1981) also formulated an equation that relates streaming potential to zeta potential, and translates the zeta potential to the applicational need. This is described by Mandaro (1987).

HYDROPHOBIC ADSORPTIONS

Theories on Hydrophobic Adsorptions

Given its abstruse nature, it is not surprising that there are several explanations for the phenomenon of hydrophobic adsorption. No challenges are posed to its reality, but there are differences concerning its cause and effects. Adamson (1982) writes, “The term “hydrophobic bonding” is appropriate to conditions wherein there is an enhanced attraction between two surfaces (as of a particle and filter) exposed to a liquid if the liquid-particle interaction is weaker than the liquid-liquid interaction.” The term “hydrophobic” implies an antipathy for water. This derives from an absence of polar groups capable of hydrogen bonding to water. It is demonstrated by an extreme immiscibility with water. Molecular structures, such as ester, and carboxylic groups that contain oxygen atoms, give rise to dipoles on account of the strong electronegativity of their oxygen atoms. The dipole/dipole and other electrical interactions account for the attractions between solid surface sites that result in adsorptive sequestrations, and also colloidal agglomerations.

Such polar features are, however, absent from hydrocarbon molecules that, nevertheless, do interact in the manner that suggests adsorptive influences. The apparent contradiction requires clarification. It will be remembered that the VDW forces that operate among hydrocarbons bereft of oxygen or other polarizing features were

hypothesized as being due to "instantaneous non-zero dipole moments" that resulted in attractions, albeit weak ones. This explains the hydrocarbon interaction as also being charge related. In this view, hydrocarbon molecules, here taken as the archetypical non-polar substances, are motivated by the opposite signs of their VDW type partial-charges to connect with other hydrophobic molecules in hydrophobic adsorptions.

An hypothesis that does not rely upon charge interactions between hydrocarbon molecules is also possible. In agreement with time-honored alchemists' observations, namely, "like prefers like," it is accepted that hydrocarbon molecules do connect with other hydrophobic molecules. The implication is that the hydrocarbon molecules' VDW attraction of the one for another is the key driver in hydrophobic adsorptions.

This seems also to be the view of Fletcher (1996, p. 3). He sees the water separating (adsorbed to) the bacterium and filter surfaces as a barrier to their coming together. Moreover, removal of the water is "energetically unfavorable," However, if either surface "has non-polar groups or patches," the resulting hydrophobic interaction will displace the water and allow a closer approach of the two surfaces. This more orthodox explanation does not rely upon charge interactions between hydrocarbon molecules. It cites the reduction in free surface energy that is an accompaniment of hydrophobic adsorptions. The implication is that this is the force that motivates the coalescence of the dispersed hydrophobic phase.

The gathering of the hydrophobic material and its separation from the water is perhaps more distinctly explained by Tanford (1980) who quotes G.S. Hartley as stating, "the antipathy of the paraffin-chain for water is, however, frequently misunderstood. There is no question of actual repulsion between individual water molecules and paraffin chains, nor is there any very strong attraction of paraffin chains for one another. There is, however, a very strong attraction of water molecules for one another in comparison with which the paraffin-paraffin or paraffin-water attractions are very slight." Thus, Tanford (1980, Chap. 5) expostulates that it is the water molecules' alliances among themselves that rejects interactions with the hydrocarbon molecules, causing a concentration of the latter: "The free energy is representative of the attraction between the substances involved. The free energy of attraction between water and hexane or octane obtained at 25°C is about –40 erg/cm^2 of contact area; the free energy of attraction of the hydrocarbons for themselves at the same temperature is also about –40 erg/cm^2; but the free energy of attraction of water for itself is –144 erg/cm^2. It is clearly the latter alone that leads to a thermodynamic preference for elimination of hydrocarbon-water contacts; the attraction of the hydrocarbon for itself is essentially the same as its attraction for water." The driving force of the hydrophobic adsorptions, then, is the reduction in free surface energy that results from the strong mutual attractive forces that manifest themselves in hydrogen bonding among the water molecules.

In the above discussion on the hydrogen bond it was stated "the water molecule is tetrahedral in shape. Each of its corners holds either a pair of electrons or an hydrogen atom. Each of the partly positive hydrogen atoms of one water molecule can form a hydrogen bond with a partly negative oxygen atom of each of four different water molecule, etc. Actually, two or three is the usual number. This process, repeated throughout the water volume, in effect creates an (imperfect) interconnected network. Thus, the molecules of water in its solid state (ice) exist as tetrahedral hydrogen bonded structures. Much of this ordered form persists even in the mobile liquid."

The hydrocarbon molecules with little affinity for the water molecules are rebuffed from intruding among these spatially, tetrahedrally-ordered arrangements. It is the network formed by the water molecules among themselves that in expelling the

hydrocarbon molecules causes their segregation. These may conjoin also to the hydrophobic areas of solid surfaces they encounter; such as of pipes or filters. In their coming together, the hydrocarbon molecules, as also the water molecules, effect a reduction in the total free surface energy. It is likely that micellar groupings are involved under the influence of area-minimizing forces.

With regard to hydrophobic adsorptions to filter membrane surfaces, Zydney (1996) describes the phenomenon as follows: "Solute-membrane interactions can occur only if sufficient energy is provided to displace the H-bonded water molecules from the surface of both the membrane and the macrosolute. In contrast, the removal of unbonded water molecules from the surface of a hydrophobic membrane or macrosolute, is energetically very favorable, leading to a very strong 'attractive' interaction between hydrophobic surfaces in aqueous solutions." Although referred to variously as hydrophobic adsorption, or hydrophobic bonding, "it really reflects the change in energy or entropy due to the dehydration of the two surfaces, and the formation of additional hydrogen bonds among water molecules.

It is possible to generalize regarding the adsorption of materials from aqueous media by viewing the adsorptive phenomenon as being in competition with the tendency of the material to remain in solution; the less water-soluble the material, that is, the less the interaction between the macrosolute and water, the easier it is to remove it from solution by the adsorptive interaction. By this measure, less ionized or nonpolar molecules are easier to adsorb because they have less affinity for the water molecules than the water molecules have for themselves. Hydrophobic adsorption assumes its importance as a particle retention mechanism on the basis of the relative strengths of the several interactions that are possible in a given situation, namely: particle to particle; water to particle; and water to water; with the last being the strongest.

Adsorption of Proteins

The strong tendency of nylon filters to remove proteins from their solutions by adsorptive sequestration is here demonstrated (Fig. 28), with specific reference to Iso gamma globulin (IgG).

The adsorption of proteins by filters has received much study (Marshall et al., 1993; Zydney, 1996, pp. 424–36.). The subject is of special interest in the biotech industry.

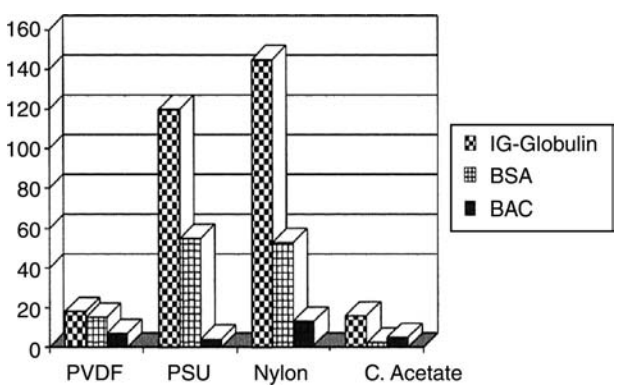


FIGURE 28 Non-specific adsorption of different membranes. *Source*: From Marshall et al., 1993; Truskey et al., 1987.

The loss of products of costly fermentations, e.g., monoclonal antibodies, is an expense to be avoided. Such loss can take place during the purification process by adsorption to filters. Yield losses due to unspecific adsorptions result in production capacity losses, and translate very specifically to market values. A gram of a monoclonal antibody product may have a market value of up to $1 million. Any milligram loss due to unspecific adsorption will accumulate over a year's production to intolerable values. It is therefore important to evaluate individual membrane polymers and filter designs to achieve the lowest possible yield loss.

A similar opportunity exists to effect the hydrophobic interaction of a microporous polypropylene membrane with lipopolysaccharidic endotoxin molecules to attain their removal from aqueous solutions. Figure 28 illustrates the differences in the avidity with which different polymeric membranes adsorb certain proteins. Each polymeric material has its own individual propensity for adsorptions. Also demonstrated is the fact that given the same polymer, different proteins adsorb to different extents. This may have utilitarian significance to bacterial fouling. The attachment of organisms to surfaces, often in the form of biofilms, is a major problem in many situations, and has, accordingly, also been widely studied (Characklis, 1990; Ridgway, 1987; Mittleman, 1998b).

However, the mechanisms of protein adsorptions have perhaps been probed even more extensively. It is the hope in this writing that the experimental elucidations of protein adsorptions can serve as a guide to the better understanding of bacterial adsorptions. As expressed by Mittleman et al. (1998b), the molecules of proteins and of organisms are complex enough to present areas of both polar and non-polar character. In the similarity of their mechanism of retention by filters may lie a commonality of their management. Encouragement is given this expectation by the findings of Fletcher and Loeb (1979) showing that marine bacteria attach most readily to hydrophobic plastics. By contrast, the smallest number attach to negatively charged hydrophilic surfaces (Zydney, 1996, p. 440).

Isoelectric Point and Hydrophobicity

Analysis of data derived from experimental studies of protein uptake by membranes indicates that sieve retention, especially of aggregated or denatured protein, is an operational mechanism (Sundaram, 1998, p. 548; Kelly et al., 1993). By far, however, protein uptake by filters is an adsorption phenomenon (Zydney, 1996). This negative charge increases in alkaline solution, whether from an increase in anion adsorption; an increase in ionization of acidic groups; or the deprotonation of basic groups, such as amines (e.g., $-NH_3^+ \rightarrow -NH_2$). The reverse behavior is true in acidic solution. Thus, as the solution pH is lowered, most amphoteric species will eventually pass through a point at which they have no net charge. The pH at which this occurs is referred to as the isoelectric point or pI. The isoelectric points of proteins range from a low of $pI < 1$ for pepsin to a high of $pI = 11$ for lysozyme. This wide range of pI reflects the impressive heterogeneity of amino acid compositions among different proteins (Zydney, 1996).

Such proteins as the serum albumins undergo hydrophobic interactions with the long non-polar chains of fatty acids that terminate in hydrophilic carboxylic acid groups. In the case of the interactions between the fatty acids and serum proteins, the opportunity for hydrophobic interaction is increased by muting the polarity of the protein. This is done by adjusting the pH to the isoelectric point of the particular protein, because at that point the electrostatic charges inherent in the amphoteric protein molecules are

neutralized. The heightened interactions of filters with proteins at the charge-free isoelectric points of the latter are taken to indicate hydrophobic adsorptions.

Conformational Changes

Truskey et al. (1987) measured protein adsorption, circular dichroism, and the biological activity of a variety of protein solutions, i.e., insulin, IgG, and alkaline phosphatase, The solutions' properties were measured before and after passing the proteins through a variety of membranes. Shifts in circular dichroism and decreases in the activity of the enzymes were determined to be the result of conformational changes of the protein structure that are attendant upon adsorption. This study showed that membranes with the greatest degree of hydrophobicity had the greatest effect on protein adsorption. This, in turn, effectuated a strong conformation with concomitant denaturation. The protein-membrane interaction resulted in the protein's exposing its internal hydrophobic sites, which were folded within its structure during its exposure to aqueous solution. This bespeaks a relationship between protein shape and function. The exposed hydrophobic sites that engaged in the membrane-protein binding, protein-protein binding, and protein denaturation were likely the more hydrophobic amino acids (see section on "The Fuoss Effect").

Opportunities may exist for the shearing of protein molecules as they negotiate a microporous membrane's tortuous passageways. A loss of the protein's properties may result. (Such, at least, are the fears of wine aficionados who decry wine filtration for the taste changes they allege to occur, presumably as a result of protein shearing.) Shearing in general is, however, seen as being less responsible for functional losses through denaturations than are the conformational changes that follow the adsorptions of proteins to polymer surfaces.

Qualitative Measurement of Protein Binding

That specific proteins exhibit differences in their adsorptive proclivities has been remarked upon. The usefulness of a means of measuring this tendency is obvious. Badenhop et al. (1970) have devised and utilized such a test method. A standardized drop of an aqueous solution of serum albumin was placed upon the surface of each of three membrane filters. The extent to which the water-drop spread was evidenced by the area of wetness. This was essentially the same for the three filters: cellulose triacetate, cellulose nitrate, and an experimental PVC. However, the spread of the albumin within the wet area differed. The visual detection of the albumin was made possible by the use of Ponceau S stain. It was found that the albumin spread along with the water over the cellulose triacetate when the bovine albumin concentration was 100 mg percent. At that concentration level, the albumin spread only very little on the cellulose nitrate and even less on the experimental PVC. At a concentration of 700 mg percent, the spread of albumin over the experimental PVC filter did not increase much, but did so for the cellulose nitrate.

This experience is interpreted as indicating the relative binding forces for the three polymers for serum albumin in aqueous solution. The cellulose triacetate is seen as exerting the least interaction; hence, the least binding and the least impeded in spreading along the membrane surface. The experimental PVC by this measure binds the strongest of the three polymers examined. The nitrocellulose binds albumin strongly, but the attractive forces at a given area are saturated at the 700-mg-percent

level, permitting the further spreading of the protein. The interaction strength of membranes is, thus, expressible in terms of its fixative properties relative to specific proteins, both in terms of the quantity of protein it can bind and in the extent of localization the bound protein exhibits.

Protein Fouling of Filters

The ease of filtering serum depends largely upon the animal species from which the serum is obtained. Several of the many components of sera add to the difficulty of their filtration. Fetal calf serum is relatively easy, and porcine serum is relatively difficult to filter. The most troublesome components are the lipids, and proteins. When their depositions and adsorptions block and clog the filters, the occurrence is called "fouling." It is perhaps curious that an anthrópomorphism is used as a pejorative term to characterize physicochemical phenomena such as molecular interactions when the consequences are not desired.

The contribution made to filter blockage by the membrane's polymeric composite is pronounced when bovine serum is the filtration fluid. Comparison was made between a 0.22-μm-rated cellulose triacetate membrane and a 0.45-μm-rated experimental PVC membrane as a filter for bovine serum. In order to minimize clogging due to particulate matter, the bovine serum was prefiltered through a 0.8-μm-rated cellulose triacetate membrane. It was found that the cellulose triacetate membrane, although characterized by a smaller mean-pore size, yielded double the throughput of the experimental PVC filter. The difference in performance is ascribed to filter interaction with the serum proteins. Protein binding "fouls" the hydrophobic (PVC) membrane and reduces the throughput. Such proteins as the serum albumins undergo hydrophobic interactions with the long non-polar chains of fatty acids that terminate in hydrophilic carboxylic acid groups. In the case of interactions between the fatty acids and serum proteins, the opportunity for hydrophobic interaction is increased by muting the polarity of the protein. This is done by adjusting the pH to the isoelectric point of the particular protein, because at that point the electrostatic charges inherent in the amphoteric protein molecules are neutralized.

COMPARATIVE DATA FROM BEER FILTRATIONS

A confirming experience is forthcoming from the brewing industry. An examination of filtration throughputs were made of a light malt liquor with that of a heavy beer characterized by an albumin content. An experimental membrane of PVC and one of cellulose acetate, both of approximately the same pore size distribution, were used. In trials using a water containing a ferric hydroxide colloid, the cellulose acetate membrane clogged at a more rapid rate; implying a less open pore structure. In apparent confirmation, the *lighter* beer (low albumin) excelled in its throughput through the hydrophobic PVC filter with its more open porosity. This indicates a greater retention due to sieving than to adsorption. However, the *heavier* beer yielded a larger throughput with the tighter polar cellulose ester membrane than it did with the more open hydrophobic PVC filter. The reversal of results was attributed to the adsorptive interaction of the non-polar experimental PVC membrane with the albumin of the heavy beer; the operation of the hydrophobic adsorption mechanism was assumed. Moreover, it is known that when beers brewed from grains that contribute albumin are "boiled," the albumin is insolublized, and membrane filtration unimpeded by undue adsorptive clogging yields more generous throughputs.

Interestingly, in the study comparing the filtration of the light and heavy beers, the clogging layer could be removed, without backwashing, by substituting the lighter beer for the heavier during the filtration. This restored the experimental PVC membrane to its pristine state. Studies on proteins have generally shown that filters composed of polar structures, such as cellulose triacetate, exhibit minimal tendencies towards protein adsorption. Nitrocellulose membranes disclose a higher tendency, possibly through the agency of hydrogen bonding. Hydrophobic filters, such as an experimental PVC, manifest the highest proclivity of these filter types to interact with proteins. Badenhop et al. (1970) experimenting in filtering heavy beers, found that the throughputs were reciprocal to the adsorption tendency. The greater the adsorptive effect, the more the "fouling" of the filter and the smaller the throughput. For proteins in general, the fouling effect, the interference with flow, depends upon the relative sizes of the protein molecule and of the filter's pores.

That hydrophilization of membranes composed of hydrophobic polymers makes them less receptive to protein adsorption is a relatively recent understanding within the pharmaceutical industry. However, it was demonstrated over three decades ago by filtration practitioners in the brewing industry. Badenhop et al. (1970) showed that coating the hydrophobic experimental PVC membrane (described above) with a hydrophilic coating resulted in a significant increase in the throughput of heavy beers that resulted from a reduction in the protein fouling of filters (Table 8). This argues for a deeper awareness of solutions to common problems that may be forthcoming from applications outside of the pharmaceutical industry.

That proteins tend to adhere to hydrophobic surfaces by way of the hydrophobic adsorption mechanism seems an established fact. It is, however, not the only mechanism for such attachments. H-bonding may also be involved. Nitrogen (and also fluorine) atoms do form H-bonds, albeit not as readily as does oxygen. Nitrocellulose is a polymeric organic ester whose structure contains three of either or both hydroxyl or nitro substituents, depending upon its degree of nitration. As Table 9 illustrates, it interacts with beer proteins (albumin) with considerable avidity. Low throughputs result, conceivably the consequences of the extent of hydrogen bonding between the partial negatively charged atoms of the nitro and ester groups and the partial positively charged hydrogen atoms of the protein molecules.

Experimental microporous Dynel's membranes also yield decreased throughputs of heavy beers. Its adsorptive coupling to proteins, possibly albumin-like, may involve H-bonding by way of its nitrogen atoms. Like cellulose nitrate it restricts the throughputs of heavy beers.

A rather crude appraisal of relative "hydrophobicity" could be ventured by comparing the percentage Moisture Regain at 65% R.H and 22°C of synthetic fibers composed of cellulose acetate, Dynel, and nylon. The less the regain, the more

TABLE 8 Comparison of Uncoated and Hydrophilic-Coated PVC-Type Membranes in Heavy-Beer Filtration

Beer A	
Experimental PVC	175
Hydrophilic Coated	263
Beer B	
Experimental PVC	241
Hydrophilic Coated	720

Source: From Badenhop et al., 1970.

TABLE 9 Comparison of Heavy Beer Throughput for 0.8-μ Nitrocellulose and Dynel Membranes

	Nitrocellulose	Dynel
	1.44	1.05
	1.55	1.25
	1.18	0.89
	1.30	1.23
	1.24	.99
	1.51	.74
Average	**1.37**	**1.03**

Filtration results are given in total liters throughput per cm^2 of filter surface area in a constant-flow-rate (30 ml/min/cm^2) filtration with a 60-psig cutoff point.
Source: From Badenhop et al., 1970.

hydrophobic the material. Nylon, known to be promotive of protein adsorption, is rated as ~3.8–4.2, Dynel at ~0.3; cellulose acetate at ~6. (Kaswell, 1953, Chap. 5). Dynel, on this basis, could be expected to reinforce its hydrogen bonding effect on protein fouling with its high hydrophobicity.

Endotoxin Removal By Adsorption

The removal of the lipopolysaccharidic endotoxin by way of the ionic interactions of positive-charged filters is discussed in the section "Endotoxin Adsorption by Ionic Interactions."

Hydrophobic Adsorption: Ultrafilters

Ultrafilters of various molecular weight cut-offs (MWCO) were tested for their abilities to remove endotoxins. The membranes were composed of polysulfone, and of cellulose triacetate (Wolber et al., 1998). Log reductions of 4–5 were obtained using filters having MWCOs of 10,000.

All known endotoxins have a hydrophobic lipid A core as well as a hydrophilic polysaccharide appendage. It is the lipid A core that is responsible for the pyrogenic activity of the endotoxin and for its hydrophobic character (Galanos et al., 1972). Its nonpolar nature reflects the 16–20-carbon chain that it contains. This nonpolar chain furnishes the site for the hydrophobic interaction between the lipopolysaccharide and the like-bonded segments of filter surfaces, the nonpolar polypropylene molecules.

Lipid A, being nonpolar, is incompatible in terms of phase separation with aqueous solutions. It spontaneously aggregates in such media to form small micelles or larger bi-layered vesicle arrangements (Sweadner et al., 1977). In these formations the negatively charged hydrophilic groups lie on the surfaces of the molecule exposed to the aqueous solutions. The core of the micelle or vesicle is composed of the hydrophobic lipid A portion of the endotoxin. The exposed anionic groups on the outside attract their oppositely charged counterions to form an electrically neutral double layer that also includes water molecules bound by dipole-dipole interactions.

Robinson et al. (1982) illustrates the lipopolysaccharides (LPS; endotoxins) pyrogenic bacterial being adsorptively retained by hydrophobic membranes through the hydrophobic interaction of the uncharged filter surface and the non-polar lipid A core of

the endotoxin. The enclosed nonpolar lipid A core establishes contact with the nonpolar polypropylene pore walls and so becomes hydrophobically adsorbed upon rupture of the charged hydrophilic pellicle. At the bottom of the hydrophobic effect is the entropically driven tendency of hydrophobic structures to interact with one another in order to reduce the area of their contact with water. The probability of this occurrence is enhanced by ultrafilters of lower pore sizes.

Hydrophobic Adsorption; Microporous Membranes

Table 10 illustrates that the adsorptive removal of the endotoxin, as measured by LRV, is indeed inversely proportional to the pore size of the membrane. As Table 10 also shows, de-aggregation of the endotoxin micelles and vesicles by the action of ethlenediaminetetraacetic acid results in the removal of the endotoxin by the nonpolar membrane but not by weakly polar cellulose triacetate membranes. These latter filters retain the aggregated pyrogens, but only because of pore size, through sieve retention.

The use of 0.2-μm-rated inverse-phase (temperature-governed) polypropylene membranes reduce the endotoxin concentration of solutions filtered through them by an LRV of 1–3. The use of a 0.1-μm-rated polypropylene membrane of similar inverse phase structure reduces the endotoxin by an LRV of 3–4. Thus, endotoxin removal by hydrophobic adsorption requires both a hydrophobic filter and intimate contact with the lipopolysaccharide (aggregate or no), as ensured by the use of small pore-size rated membranes. The microporous polypropylene membranes produced by the temperature-governed phase inversion process meet both requirements (Hiatt et al., 1985).

Practical Implications of Adsorptive Sequestration

The loss of permeability with continuing filtration has been probed by several investigators. The findings were expressed in mathematical equations depicted as plotted curves whose analysis led the experimenters to conclusions. As an example of such efforts, Johnston constructed log/log plots of a constant pressure filtration wherein volume filtered over time, V, vs. time, t, was express for each of four different circumstances: (a) cake filtration, (b) intermediate blocking, (c) standard blocking

TABLE 10 Influence of Pore Size and Endotoxin Aggregation State on Endotoxin Adsorption to Filters

Polymer type	Pore size (μm)	Endotoxin[a] aggregation state	Endotoxin LRV
Polypropylene	Prefilter	DI	0.1
	0.2	DI	1-3
	0.1	DI	3-4
Cellulose acetate	0.2	DI	0.1
	0.025	DI	3.0
Polypropylene	0.2	EDTA	1.0
	0.1	EDTA	1.0
Cellulose acetate	0.2	EDTA	0.1
	0.025	EDTA	0.1

Note: Deionized water (DI) solutions of endotoxin are mixtures of vesicular and mlcellar structures. 0.005 M EDTA solutions have only micellar structures.
Source: From Badenhop et al., 1970.

(d) complete blocking (Johnston, 2003, p. 104). In this, the author's writing, conclusions derived from Bowen et al. (1967) are set forth.

Bowen et al. (1976) investigated the mathematics of particle capture within pores of fine capillaries. Although complex, the key parameters can be summarized. The first is a reduced Peclet number, γ, which is a dimensionless parameter containing the average fluid velocity, V_m, the pore radius, R, the solution diffusion coefficient of the particle, D, and the length of the capillary, x, and is defined as:

$$\overline{\gamma} = \left(\frac{D}{2V_m R}\right)\left(\frac{x}{R}\right)$$

The two terms in parentheses represent two effects. The first is the competition between diffusion, tending to move the particle toward the wall, and convection tending to carry the particle down the capillary and out the filter. The second, expressed by the ratio x/R, can be regarded as the number of chances that diffusion will have to carry out its task.

Bowen et al. (1976) present an interesting treatment of retentivity, or of its equivalent, the collection efficiency that is at work in adsorptive sequestrations. They plot this parameter as a function of γ for different values of the particle–wall reaction constant K. When K is infinitely large, all particles are captured at all fluid velocities. When K is simply large (i.e., a strong attractive interaction existing between the bacterium particle and the pore wall), then a range of conditions is present wherein the capture efficiency will be less than unity; an organism could "slip through". The equation predicts that as the mean fluid velocity, Vm, increases, γ will decrease, as will the capture efficiency. Thus, higher pressures which increase Vm and produce faster filtration times, also decrease the absolute retentivity of a membrane that operates by adsorptive sequestration. Furthermore, for a given bacteria/wall interaction and a fixed set of flow conditions, the capture efficiency is independent of the total volume of fluid passed. Thus, a membrane operating by this mechanism at bacterial levels low enough to yield absolute collection efficiencies will be independent of the total challenge per square centimeter of membrane surface; in contrast to the membrane operating by sieve retention alone, wherein the total bacterial challenge is important (presumably in relation to the number of pores present in the filter).

By contrast, neither the thickness of the membrane, the concentration of particles, the fluid velocity, nor the applied pressure will affect the selectivity of a surface (filter cake), sieve-retaining filter toward bacteria. Only the pore size is important, and if all pores are equal in size to, or less than, the organism, the filter will be absolutely retentive. However, all commercial membrane filters have pore sizes distributed about some mean. Thus, it is conceivable that there exists at least one set of pores sufficiently large to allow the passage of a single organism. Hence, the rationalization that if the number of organisms filtered per square centimeter of membrane approaches or exceeds the number of pores per square centimeter, the membrane is unlikely to prove absolutely retentive. For sieve capture, the critical parameter for testing the membrane's absolute retentivity is:

$$\text{Total final challenge} = \frac{\text{No. of organisms filtered}}{\text{Area of filter}} = \frac{\text{Total CFU}}{A}$$

The practical implications of the pressure differential employed in filtrations merits being repeated. Higher applied differential pressures will result in higher rates of flow. The price may, however, be paid in terms of reduced filter efficiencies and shorter service lives.

(1) The equation indicates that as the mean fluid velocity, V_m, increases, γ will decrease, as will the capture efficiency. Thus, higher pressures which increase V_m and produce faster filtration times, also decrease the absolute retentivity of a membrane that operates by adsorptive sequestration.

The inverse relationship of differential pressure with retention, presumably not an expression of sieve retention, fits in with adsorptive sequestration. This was demonstrated by Tanny et al. (1979).

(2) For a given bacterium/wall interaction (i.e., adsorption) and a fixed set of flow conditions, the capture efficiency is independent of the total volume of fluid passed. Thus, a membrane operating by this mechanism at bacterial levels low enough to yield absolute collection efficiencies will be independent of the total challenge per square centimeter of membrane surface; in contrast to the membrane operating by sieve retention alone, wherein the total bacterial challenge is important, presumably in relation to the number of pores present in the filter.

The statement suggests that retentions by adsorptive sequestration would be independent of the bacterial dilution, whereas sieving which relies upon pores, would not give independent results. Jornitz and Meltzer (2001), commenting on work reported by Grant and Zahka (1990) using silica particles, and by Roberts et al. (1990) using latex beads, state that dilute challenges would not be problematic to adsorptive captures, but would be a worst case scenario for sieving. Experimentation establishing the dilution effect of organism suspensions have yet to be performed.

(3) By contrast, neither the thickness of the membrane, the concentration of particles, the fluid velocity, nor the applied pressure will affect the selectivity of a surface (filter cake), sieve-retaining filter toward bacteria. Only the pore size is important, and if all pores are equal in size to, or less than, the organism, the filter will be absolutely retentive. Indeed, this is the definition of an absolute filter. It does assume incompressible particles.

(4) An initial high concentration of organisms is not essential to less-than-absolute retention. The ultimate level of the total viable organism challenge is the important factor. Thus, a sufficiently large volume of low bacterial concentration could, in principle, predispose toward non-sterile filtration where sieve retention is the sole capture mechanism, and where all the organisms are not necessarily larger than the largest pores.

It is assumed that "all the organisms are not necessarily larger than the largest pores" equates with a situation wherein the organism challenge confronts a filter with a pore size distribution. As detailed below, Jornitz and Meltzer (2001, pp. 562–4), commented on work reported by Grant and Zahka (1990) using silica particles, and by Roberts et al. (1990) using latex beads. They state that dilute challenges would not be problematic to adsorptive captures, but would be a worst case scenario for sieving. Detailed experimentation investigating the dilution effect of organism suspensions have yet to be performed.

The practical implications of the pressure differential employed in filtrations merits repeating. Higher applied differential pressures will result in higher rates of flow. The price may, however, be paid in terms of reduced filter efficiencies and shorter filter service lives.

SUMMARY

The appellation "sterilizing" filter, conferred by the filter manufacturer on the basis of integrity test values correlated with organism retentions, qualifies a filter for trial. Whether its promised potential is fulfilled in a given processing context can be judged only by accomplishing filter validation studies utilizing product-related bacterial

challenge testing. The meaning of "sterilizing filter" is relevant only when applied to a specific filter, with a particular fluid, and a defined microbial content under stipulated conditions. Extrapolations to other conditions, however circumspect, that fail to recognize that it is the unique attributes of the individual filtration that are paramount in the ability to provide a sterile effluent, are inherently unwarranted.

It is now appreciated that organism retention by a filter is governed by an interaction of influences more complex than the proportion of pore and organism sizes alone. In any case, the physical and chemical properties of the suspending liquid contribute to adsorptive and impactive arrests and are certainly capable of altering filter pore and microorganism sizes. Consequently, integrity test measurements, based upon the assumption of both fixed pore and organism sizes, cannot unerringly signal the reliability of organism removal by size exclusion. This reduces their heretofore assumed uniqueness as categorical indicators of filter validations (Jornitz et al., 2002). Their critical and decisive function as arbiters of filter integrity remains unimpaired.

The misleading imputation of organism (particle) retentions as arising solely from size exclusion origins should be abandoned. Adsorptive/impactive mechanisms of organism retention compel consideration.

The definition of "sterilizing" filter becomes restricted to that filter which, under specific operating conditions, removes all organisms of interest known to be present in the given preparation. The achievment of sterility is decided in terms of the specific organism(s) whose complete removal is validated by documented experimental evidence. The attestation requires positive outcomes to the isolation, cultivation and measurement of the target organism(s). As in the case of *B. diminuta,* the subject organism(s) may be assumed to be a model for others whose presence or absence is not assessed. Such assumptions may prove unjustified. Where the sterility of drug preparations is involved, assumptions will not suffice. Validation is necessitated.

Increased dependence now devolves upon bioburden studies, recognized to be under strong limitations themselves. Nevertheless, bioburden investigations, demanding though they be, are required for the validation of sterile filtrations. One must be able to identify and quantify the organisms whose removal is the object of the filtration exercise.

The attainment of a sterilizing filtration requires validation of the filter and of the filtration process. Documented experimental evidence is essential to the process.

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6

Microbiological Considerations in the Selection and Validation of Filter Sterilization

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BACKGROUND

Treatises on the filtration of liquids with the objective of achieving a sterile effluent are typically replete with detailed discussions of the physical and chemical characteristics of filters and the various mechanisms at play in retention. Rarely does one find a careful consideration of the microbiological considerations of filtration in spite of the simple truth that the retention of microorganisms is a nearly all-consuming component of the filter validation exercise. It must also be said that "sterility" of the effluent is arguably the aspect of filtration that receives the greatest regulatory scrutiny both in product dossier review and in plant inspection.

The purpose of this chapter is to examine microbiological considerations of filtration in depth. This contrasts with the typical treatment of microbiology in filtration science, which is sad to say rather superficial. Everyone involved in aseptic validation knows that a model organism has been chosen and that it is *Brevundimonas diminuta* (ATCC-19146). Knowing this, they will be aware that FDA has defined a proper challenge as not less than 1×10^7 colony forming unit (CFU) of this organism per square centimeter of available filter surface area. It is certainly tempting to believe that there is some significance to the selection of 10^7 CFU per square centimeter of filter surface, and that perhaps it relates in some manner to the 10^{-6} "sterility assurance level" that applies to physical sterilization. However, the origins of the 10^7 CFU/cm^2 challenge level are not completely clear, perhaps their genesis relates in part to the notion that a concentration one order of magnitude greater than 10^6 would result in a satisfactory "worst case" condition. "Worst case" in the realm of microbiological validation of filters can be a very difficult condition to define, and in the author's opinion careful scientific analysis regarding appropriate microbial titers for the challenge have been lacking.

STERILIZING GRADE FILTERS—A MICROBIOLOGICAL PERSPECTIVE

Filter sterilization can be strictly defined is the complete elimination of viable organisms of any species from a fluid. A liquid, containing suspended microorganisms would be rendered free of contaminating microbes by separation of these organisms from the

liquid. Presently, filters with a 0.2 or 0.22-μm pore size rating are considered sterilizing grade filters, although FDA defines a sterilizing filter as one that can retain the aforementioned 10^7 CFU/cm^2 of effective filter area challenge with *B. diminuta* (ATCC # 19146) a result of no recoverable CFU in the effluent.

It seems appropriate to ask if this definition is really valid from a scientific perspective. First, one might ask if *B. diminuta* is indeed a representative model organism, the answer would appear to the author to hinge upon the objective of the validation test. If the desire is to conduct a risk based analysis to determine if the filter will retain, with a high level of safety, typical bacterial and mycological flora likely to be present in a fluid prepared under reasonably controlled compounding conditions, the answer in most cases would be yes. However, if the challenge with *B. diminuta* is intended to demonstrate absolutely and without equivocation the ability of the filter to produce a sterile effluent, defined as an absolute the answer would quite obviously be no. Actually, it might very well be impossible to claim that a 0.2-μm filter could even be defined unequivocally as a bacterial retentive filter.

For example, organisms of the family Rickettsiaceae number among them numerous important human pathogens (a pathogen being defined as an organism known to produce disease). Among the diseases produced by members of this family are typhus, Rocky Mountain spotted fever, trench fever, and psittacosis (*Coxiella burnetti*). Fortunately, the majority of organisms in this family would not be expected to survive in dry powder active ingredients or excipients, nor in the typical compounding environment. However, some *Coxiella* spp. can survive heat and drying and all of these organisms can survive at –70°C for extended periods of time and in lyophilized materials. These organisms lack rigid cell walls and as a result are pleomorphic, which means they are variable in size and shape. These organisms can be found in nature in the 0.3- to 0.6-μm size range which is to say that they could be at least as difficult to filter as *B. diminuta*.

If we leave the realm of true bacteria when can find another class of highly pleomorphic prokaryotic organisms-mycoplasma. These organisms lack the true cell wall characteristic of true bacteria and typically range in size from 0.2 to 0.8 μm, although smaller examples among the family mycoplasmataceae have been reported. In the last 10 years the filtration of mycoplasmas has become an issue, particularly with respect to the manufacturing of biotechnology and biological products. Largely, this is because mycoplasma can be endemic in some of the ingredients used in the preparation of cell culture and tissue culture media. Studies published by Sundaram et al. clearly indicate that if a challenge level of 10^7 CFU was used in conjunction with many commercial 0.2-μm filters complete retention will not occur. This led to the suggestion that perhaps filters in the range of 0.1-μm pore size rating should be considered sterilizing grade filters rather than 0.2 or 0.22 μm pore size rating filters.

If we move not only away from true bacteria but in fact away from prokaryotes in general we can identify no shortage of organisms can still fall under the category of microbes which would not be absolutely retained by even 0.1-μm filters. These organisms are of course viruses. Viruses are obligate intracellular parasites which lack even the most basic of metabolic capability. Viruses in fact are not arguably alive at all until or unless they are in contact with a cell which is permissive to infection by a particular viral species. Unless they are in the process of replicating a virus is in fact biologically inert.

Because virus are not possessed of metabolic apparati, they have a remarkably simple and efficient structure. In their most basic configuration they can consist of nothing more than a rather small single strand of genetic material and a handful of structural proteins. For example, the enteroviruses, a collection of several hundred distinct viral serotypes, contains among its numbers a significant array of human and

animal pathogens. Two notable examples are the polioviruses and their close relatives the coxsackie viruses as well as the foot and mouth disease virus. The reader may recall a significant outbreak of foot and mouth disease virus in Great Britain in 2001 and 2002 which resulted in tremendous public health concerns and result in billions of dollars of loses in the food animal industry in that country. These viruses consist of a single strand of RNA with a total size of ~1×10^6 D, but comparison a typical bacteria would contain genetic material (double stranded DNA) with a mass of > 10^{12} D. These viruses have an icosahedral shell made up of four structural proteins and a small nucleic acid associated protein. Not surprisingly, these viruses can be quite small in fact on average they are roughly 25–30 nm in diameter. A nanometer is 10^{-9}m or 1000 times smaller than a micrometer. Thus, it seems unlikely that a filter with a mean pore size rating of 0.2-μm would fare well against a challenge by Enterovirus, at least in terms of separation by sieve retention.

There are smaller and larger viruses than the Enterovirus example. The largest virus, the poxviruses are in the range of 400 nm. Other viruses known by name to the reader would include Herpes viruses, which are typically 150–180 nm in diameter and the human immunodeficiency virus which is just slightly smaller. However, none of these viruses would be expected to be efficiently retained by sieve mechanisms by 0.2-μm filters. During my years as a research virologist I had occasion to use 0.22-μm nylon 66 or cellulose acetate filters to clarify solutions containing up to 10^8 plaque forming units of various species of poxvirus per mL. On average the virus titer was notably reduced, in some cases by 50–90%, which of course meant that the post-filtration titer was still 10^7 plaque forming units or more. The filtered solutions were probably bacteria free, but were obviously not virus free and therefore not sterile. So, in the work described above a filter often defined in the pharmaceutical industry as sterilizing grade was used to clarify a solution of virus and to remove bacteria specifically because it would not remove viruses. Thus, a "sterilizing grade filter" can be used specifically because it will not yield a sterile effluent under some use conditions.

Actually, medical microbiology played a central role in the development of filtration and in the use of filters was critical to the discovery of viruses. In the late nineteenth century alternatives to heat sterilization of bacterial media were sought and it occurred to scientists that it could be possible to develop filters which would retain bacteria leaving only sterile effluent. Chamberland and Pasteur working independently developed unglazed porcelain filters and efforts to develop cellulose fiber filters proved unsuccessful. By 1891, bacterial retentive filters using Kieselguhr (diatomaceous earth) had been developed. Shortly thereafter in 1892 Iwanowski demonstrated that the agent that caused tobacco mosaic disease retained infectivity even after a solution containing this agent passed through a bacterial retentive filter. In 1898, Loeffler and Frosch demonstrated that the agent which caused foot and mouth disease in cattle was also "filterable." Initially the organisms we now know as viruses were called "filterable agents" and by the early twentieth century it was understood that all three of the major groups of viruses, plant, animal and bacterial, were small enough in size to pass through bacterial retentive filters.

During our lives we have witnessed the emergence of a new category of disease producing entities which are smaller and simpler than viruses—in fact much smaller and simpler. These entities are called prions and are now widely accepted as the etiological agents of an array of transmissible spongiform encephalitides. These entities lack even genetic material in the form of RNA or DNA and are in fact proteins with the ability to replicate in brain tissue. These are fairly rapidly progressive diseases which are uniformly fatal. Obviously, "sterilizing" grade filters would not retain them, even if we define

sterilizing grade as 0.1 μm. There could of course be new categories of disease caused by similar agents, or by viruses or even by components of microbes that have not yet been detected. The world of infectious diseases is a constantly changing, or one might rightly say evolving one, in which new discoveries are often made.

With the foregoing in mind, it can be concluded that one cannot merely select a filter of 0.2 μm or "better" in the jargon of validation and be certain that the result will be a sterile effluent under all possible conditions. This holds true even if that filter is subject to the standard microbial challenge test at a "worst case" microbial level of $> 10^7$ CFU/cm^2 effective filter area. This holds true even if the challenge test was conducted in a solution that mimics product and the challenge level was slightly higher to ensure the "most challenging conditions" again in jargon of validation.

The ability of viruses and certain families of bacteria to penetrate filters has been understood by research scientists for a very long time. However, the reader could reasonably ask if there is any evidence of filters having been penetrated under actual use conditions. Leo et al. (1997) reported that *Ralstonia pickettii* (formerly *Burkolderia pickettii* and *Pseudomonas pickettii*) had in a manufacturing setting penetrated 0.2-μm filters. Articles by Sundaram et al. (1999) reported that 0.2-μm filters might not reliably remove "diminutive" bioburden organisms and that 1 μm could provide a safer solution.

The author is also aware through personal communications of studies in which bioburden organisms (bacteria) have penetrated 0.2-μm filters at concentrations of 10^7 CFU/cm^2 effective filter area. It is not surprising from a scientific perspective that under some conditions "diminutive" bacteria can penetrate "sterilizing" grade filters.

A RISK-BASED APPROACH TO FILTER EVALUATION

It seems obvious after considering the full spectrum of disease producing microorganisms that defining a filter by virtue of its pore size rating alone, as a sterilizing grade filter, is too simplistic. The traditional method of filter evaluation is akin, and probably intellectually related, to the overkill approach often chosen for the validation of physical sterilization processes. However, filtration and moist heat sterilization are two very different processes and technically there are no significant parallels. Resistance of moist heat biological indicators, for example, has no real relationship to size of a filter challenge organism although both resistance and size are thought to be important to achieving "worst case" challenge conditions. The reason that no relationship exists is that filters retain, they do not kill, whereas physical sterilization methods kill but leave remnants of the killed (as defined by the ability to reproduce) behind. Therefore, it can be said the filtration works in a manner that is almost exactly the opposite of physical sterilization.

What is very clear is that in both physical sterilization and filter sterilization the nature of and number of the pre-sterilization bioburden is critical. In the case of physical sterilization by moist heat or radiation the fact that microbial remnants including toxins could be left behind as contaminants (although most toxins are denatured by heat sterilization). In filter sterilization, the type and quantity of the bioburden are also vital to performance and hence mitigation of risk.

The brute force approach to filter evaluation can and has created the impression that because filters can retain heroic numbers of the model challenge organism, bioburden control is only moderately critical. It is often thought that provided a solution is beneath a product's acceptable level of endotoxin post-filtration, pre-filtration bioburden must have been adequately controlled. Actually, bioburden control may be the

most important single risk-mitigating factor in filter sterilization. Particularly since the selection of a sterilizing grade filter of 0.2 μm or smaller is a given and choosing a filter of appropriate effective filter area relates to volume to be filtered, desired flow rate and of course, bioburden.

An alternative approach to proving the efficacy of a filter sterilization process is to demonstrate that bioburden is consistently controlled to a pre-determined safe level. EU (CPMP/QWP/486/95) provides clear guidance by setting a recommended pre-filtration bioburden level of 10 CFU/100 mL. The FDA on the other hand appears to allow more flexibility stating only that pre-filtration bioburden should be controlled. It is self-evident that the lower the bioburden the lower the risk of an effluent containing microbial contaminants. From this perspective alone the EU recommendation, which is the same as the compendial limits for water for injection, seems appropriate. It is important to note that 10 CFU/100 mL is a challenge level applied to the sterilizing filter, thus this level could be achieved through the use of pre-filtration if necessary. While it may not be possible to achieve 10 CFU/100 mL in every case, it is reasonable to think that it may be possible in many if not most cases with appropriate care, process knowledge and physical controls in place.

BIOBURDEN CONTROL

Given the central importance of bioburden control to risk mitigation and process control it seems reasonable that microbiologists should carefully study all aspects of product formulation upstream of the filtration step. The purpose of this study is to ensure that appropriate controls are in place to ensure that microbiological control is sufficient to ensure that a low target bioburden level can be attained. Among the principle issues that must be considered are:

1. *Facility design:* Including proper zoning of clean rooms, barriers or isolators, personnel and materials flow, decontamination and sterilization of vessels and utensils, decontamination of environments, gowning requirements, utilities services, and temperature and humidity control.
2. *Equipment selection:* Ensuring that materials are easily cleanable and can withstand repeated and rigorous antimicrobial treatments.
3. *Definition of control conditions:* Establishing frequencies of antimicrobial treatments to process equipment and the facility, putting in place control points where risk of microbial contamination is found to be high. Controls could include refrigeration, pre-filtration and definition of appropriate hold times.
4. *Sampling and monitoring:* Microbiological monitoring of the environment and sampling of product at various steps in the process to ensure that controls are working as they should. It is critical to note that all monitoring and sampling must be done under aseptic conditions to ensure that the collected samples are representative and remain as unaffected as possible by human intervention during the sampling process.
5. *Microbiological evaluation of incoming active pharmaceutical ingredients and excipients:* It is often critical to know that the producers of bulk pharmaceutical actives and excipients have adequate process controls in place to minimize bioburden and that they are able to package and ship the product in a manner that minimizes contamination risk. Among points to consider are the nature of the chemicals, for example, are they inherently antimicrobial or would they support survival or even growth? It is also critical to consider how chemicals are packaged and bagged,

microorganisms have been found between layers of bags and corrugate packaging can harbor microbes. Finally, the integrity of chemical containers and their ability to withstand the shipping process without loss of integrity should be considered.

Control of bioburden at the compounding level is too often thought to be non-critical in terms of microbiological safety, or "sterility assurance." This is often defended by the seemingly logical argument that validation of the filter system and process is accomplished under worst case conditions. It seems logical, at least superficially, that if a filtration process is capable of retaining 10^7CFU/cm^{2}controlling bioburden to very low levels is unnecessary and perhaps wasteful of time. In cases where the characteristics of the product ensure that microbiological risk is minimal perhaps this is true. However, some scientists have been surprised to learn that even products that appear upon inspection to be very low risk in terms of microbial growth or survival can under normal production conditions have far higher than expected bioburden levels.

ESTABLISHING A BIOBURDEN ACCEPTANCE CRITERION

The previously mentioned recommendation of a 10 CFU/100 mL target level for pre-filtration bioburden may work in many cases, but certainly not all cases. However, it is important to consider the inherent variability of microbiological analysis. Most microbiologists would agree that a reasonable and therefore acceptable level of variability in microbiological analysis is on the order of 30% or more. As a note of caution one should not assume that the rapid microbiological methods, which are becoming increasing important to industry, are inherently more accurate, precise or reproducible than traditional growth and recovery based microbiological methods.

Given levels of variability inherent in microbial analysis and considering also that microorganisms are not homogeneously distributed in most environments or materials it is not appropriate to, for example, set a target level of X CFU/mL and reject the material at a level of X+1 CFU/mL. The CFU is not a direct measure or count of microorganisms present. The CFU is exactly what its name implies; it is a "unit" of microorganisms capable of growing into a single visible colony on solid medium. Said unit may consist of 10 viable cells or it may contain 30 viable cells. It is quite possible that a large unit of cells could break into two or more smaller groups and manifest as two or three CFU rather than one. Therefore, some latitude should always be given regarding microbiological limits. The recently implemented harmonized compendial microbial limits test (USP <6 1>, <6 2> and <1 1 1 1> state that in the case of an established level of 10 CFU the maximum acceptable count should be 20 CFU. Similarly if the established target level is 10^3 CFU the maximum acceptable count should be 2000 CFU. In general, a membrane filtration approach to bioburden testing will be easier for most users to implement and with the majority of products more suitable than direct plating methods in the evaluation of pre-filtration bioburden

FREQUENCY OF BIOBURDEN SAMPLING

The frequency of sampling should also be a risk-based decision predicated upon product and process knowledge. Products that are known to be inherently low risk in terms of microbial survival or proliferation may not require testing on a lot-by-lot basis. Products that are extremely antimicrobial or which contain a preservative system may not require

routine testing at all. On the other hand, products that are supportive of microbial growth should be tested on a lot-by-lot basis.

The importance of proper physical controls for bioburden cannot be over-emphasized. Bioburden sampling is generally a non-specific growth and recovery study with the aim of generating a total aerobic count. As such this is a general screen and is unlikely to recover all microorganisms that could be present in the material. Therefore, the microbiologist is well advised to focus upon the implementation of controls that will reduce risks from all microorganisms that could be present in the product or the environment. One must always remember that even a count of zero CFU does not mean the absence of contamination but rather that any contaminants present were not recovered. Zero recovery certainly implies very low risk, but it is impossible to establish that zero means no risk at all.

FURTHER CONSIDERATION ON THE MICROBIAL CHALLENGE

Mechanisms of Microbial and Particulate Retention

The mechanics of membrane filtration are too complex to discuss in detail in a brief chapter focuses in microbiology, however no discussion of filtration process development, control of validation can be complete without some consideration as to how filters retain microorganisms. Although the image that most individuals have is one of microorganisms being separated by uniformly spaced pores of identical size, much like insects on a window screen, reality is considerably more complex.

Actually, there are several mechanisms by which organisms are removed from a process stream by filters. The most important is sieve retention, also known as "direct interception" or "size exclusion." Particles are restrained from passing through the filter because their size is larger that the restrictive cross-sections of the convoluted filter pores through which the conveying fluid flows unimpeded.

However, it is possible for organisms too small to be removed by sieve retention to become attached to a pore surface by adsorptive effects. In general these absorptive effects are related to electrical charges. They may originate from full ionic charges, or from molecular structures characterized by dipoles, whether permanent or induced. Most frequently, the partially charged atoms of such structures result from the unequal sharing of the covalent bonding electrons. These find expression in any of several different bonding interactions. Among the better known of these are hydrogen bonding, the van der Waals forces, and hydrophobic adsorptions. As is well known, oppositely charged entities attract one another to form adsorptive interactions. It is such electrical bonding that attaches the partially charged atoms on an organism surface to molecular structures of opposite charge sited on the surface of a filter. The molecular structures of both organisms and filters are complex enough to provide ample sites for such mutual adsorptive bondings.

Microorganisms may be found as free living single cells in nature, but it is far more common for them to be attached together in clumps. The surfaces of microorganisms may be inherently sticky as a result of capsular structures, which can provide environmental advantages in terms of protection against damage and in repelling a hosts immune response. Fortuitously, bacteria smaller than *B. diminuta* would be a minority among the bioburden found in typical process streams. Also, beneficial is the fact that under no circumstances should the bioburden approach the filter challenge level of 10^7 CFU/cm^2 of effective surface area. Historically, the assumption has been made that viruses are not a significant risk factor in the filter sterilization of drugs and biologics. The prevailing data

may bear this assumption out, but given the absence of analytical data it is not possible to discern whether there have been adverse reactions that could be attributed to virus, microbial toxins, diminutive bacteria or non-bacterial prokaryotes that could have passed through a filter.

Microbial Challenge Testing of Filters

Filters used in the production of sterile products are always ≤0.2-μm pore size rating. This physical characteristic is always a primary requirement for a filter to be considered acceptable for use in the production of a sterile product stream. Further confirmation of a filter's suitability is established by means of the challenge with *B. diminuta* discussed previously in this chapter. The FDA has defined a proper challenge as one that confronts every square centimeter of the available filtration area with 1×10^7 CFU of this model organism. As previously mentioned, it is important to consider that this challenge test does not confirm the unequivocal ability of a filter to produce a process stream that is always free of microbial contamination. The true mission of the filter is to adequately abate microbiological risk in terms of infection of the end user, absolute sterility being impossible to establish by this or any other widely applied test including the so-called "sterility test." The sterility test is of course, itself often dependent upon filter sterilization.

SAMPLING

The proper management of the sampling technique is itself an important subject deserving of a full but separate discussion. The size of the effluent sample or the number of samples that are analyzed should be large enough to offset the heterogeneity that is typical of suspensions, and to yield a count of some reliability to be made. The suspended organisms will over time adsorb to the surfaces of the sample-container regardless of its material of construction, and quite possibly to each other. The bacterial titer can be diminished by binding of organisms to each other and to surfaces and also by die-off. If the analysis is not to be performed promptly, refrigerated storage must always be utilized to prevent, or minimize organism growth or loss in viability. The size of an organism changes as it develops through its growth stages during its cultivation. Typically, the growth curve for most organisms placed in a new environment extends through three phases. During the first or lag phase the organism adjusts to its environment and particularly to the nutrient conditions to which it is exposed. There is an increase in the individual cell size before there is an increase in numbers as the individual cell activates the enzymatic apparatus necessary to replicate its genetic material, structural components and metabolic machinery. In the second or exponential growth or log phase, attached cells begin to divide, and under appropriate growth conditions a logarithmic increase in cell population occurs. The cell numbers increase more rapidly than does the cell mass. Thus the cell numbers are increasing, but the size of the individual cells typically are decreasing. Newly replicated cells are smaller than their parent cells and as the nutrient supply diminishes cells are unable to continue to replicate at a logarithmic rate. The rapid build up of toxic waste materials during the so-called "log phase" of population expansion further slows growth. There follows the third "stationary phase" wherein the number of new cells equals the number that are dying. In the later stages of this phase there are increasing amounts of dead organisms and cell debris. The early stages of the stationary phase are, therefore, optimal for the selection of bacteria for use in the

challenge of filters. Procedures for enumeration of the culture on solid medium and microscopic evaluation of the bacteria follow standard microbiological laboratory practices.

Given the difficulties associated with the preparation of cultures of *B. diminuta* that are at the proper concentration and also within the most desirable size range filter challenge testing is often contracted to specialized laboratories which possess experience in the conduct of these assays. While the methods employed are by no means beyond the capability of most microbiological analytical laboratories, they are sufficiently unique that they are often left to laboratories operated by the filter manufacturers themselves. This has let to concerns that in many, if not most, cases, the filter vendors are allowed to determine the adequacy of their own product's performance. In general, when the challenge testing is contracted to an outside laboratory to perform, the customer's microbiologist(s) should conduct a through scientific evaluation and compliance audit of the facility and its personnel.

Challenge tests are conducted using actual product if possible, if this cannot be done because of the inability of the organisms to survive in the solution than a placebo as similar as possible to the product is used in lieu of actual product. This may not in all cases be a perfect test since the tonicity of product may change the size of bacteria in the challenge culture. However, realistically this is acceptable since the test is being conducted in conditions that approximate those that will apply in actual production. Another consideration that must often be included in the challenge test is filtration time since in products that are supportive of microbial growth population increases are theoretically possible during processing. If microbial proliferation in product is a concern it may be advisable to filter the product first into a sterile holding tank to mitigate risk, and then to further manage risk from aseptic connections a second filter can be used immediately upstream of the filling system.

The acceptance criterion applied to the challenge test is that no growth of *B. diminuta* can be recovered from the challenge test effluent. Realistically, this means that the actual retention required to pass the challenge test is often slightly greater than 10^7 CFU/cm^2. It is considered that a filter passing this challenge test is in fact a "sterilizing" filter.

Actually, this conclusion is based upon assumptions that are arguably too general. That the filter performed as desired under given circumstances does not ensure that it will necessarily act similarly with other types of organisms, or even with the same organism under different filtration conditions. The earlier belief that sterility resulted exclusively from the particles being too large to negotiate the filter's pores is now known to be far too simplistic. Each filtration is an individual expression of several factors whose balanced influences govern the outcome of the organism/filter interaction.

FACTORS THAT MAY INFLUENCE RETENTION

The retention of an organism by a filter represents a nexus of many interdependent factors. These include the filter pores, their numbers and size distribution, and restrictive diameters; as also the types and sizes of the organisms, their numbers, sizes, and structural make-up. It is the matching of the sizes and shapes of the pores and organisms that is the determinant in the sieve retention or size exclusion mechanism. It is axiomatic that a particle larger than a pore cannot penetrate it unless compressively deformed by excessive differential pressures. Although membranes are classified in terms of single pore size ratings, they are actually characterized by pore size distributions, albeit usually

of an unknown magnitudes. Thus, it is conceivable that an organism that could be retained by a pore of one of the sizes characterizing the distribution would not be retained should it confront a pore at the large end of the size distribution curve. Absolute retention is possible only when the smallest particle of the particle size distribution is larger than the largest pore of the pore size distribution. Given the usual application of filters, of unknown pore size distributions, to the removal of organisms of unknown size distributions, it would be presumptuous, and misleading to declare that a particular filter will be absolute in its action.

Sieving, the chief mechanism of particle removal, can be reinforced by the adsorptive sequestration of the particles. The electrical charges on the surfaces of the organisms and filters manifest mutually attractive forces. The result is the arrest and removal of the organisms as the fluid flows through the filter pores. The polymeric composition of the filter matrix in terms of its polarity determines its surface charge and, hence, its tendency to undergo adsorptive effects. However, the physicochemical nature of the suspending liquid can so modify the electrical attractive and repulsive forces by its ionic strength, pH, surfactant content, etc. as to promote or hinder adsorptions. Thus, the same organism and filter may interact differently in solutions of various compositions; a desired organism removal may or may not result. An example of such an occurrence was noted by Bowman et al. (1960):A 0.45-μm rated mixed cellulose ester membrane could be used to sterilize a preparation by the recovery bacteria in sterility testing. However, the addition of penicillinase to the same preparation enabled bacteria to migrate through the same filter. This proteinaceous enzyme preempted the adsorptive sites of the filter, preventing the sequestration of the organisms. That adsorptive influences were at work was shown by the eventuation of a sterile effluent from the penicillinase preparation when a 0.22-rated membrane was employed. Sieve retention was the effective mechanism when a tighter membrane was used.

The differential pressure is especially influential in its effect on adsorptive interactions. The higher it is, the faster the liquid flow and the shorter the residence time of the particle within the pore passageway. This reduces the particle's opportunity to encounter the pore wall and, thus, minimizes the likelihood of adsorptive sequestrations. Intriguingly, as reported by Mouwen and Meltzer in 1993, there is reason to believe that the concentration of organisms, aside from their total numbers, can influence the attainment of sterile effluent.

Other chemical conditions present during filtration can have an influence on organism removals. For example, the viscosity of a solution may be high enough to prevent an organism within the liquid stream from reaching an adsorptive site on the pore wall before it is carried out of the filter by convective flow. Given the reciprocal relationship of viscosity and temperature, the opposite effect can result when the same filtration is performed with the liquid preparation at a higher temperature.

Also, the ratio of organisms to pores can strongly affect the rates of flow and their consequences in terms of throughputs and filter efficiency. Thus, even the effective filtration area may impact on the likelihood of obtaining sterile effluent. In fact, there continues to be some debate as to whether higher bacterial titer challenge levels actually represent worst case conditions or whether more dilute concentrations may actually prevent rapid clogging of pores which would be expected to increase apparent filtration efficiency.

The sterilization of a fluid by a filter is, as can be seen from the preceding brief overview is exceedingly complex and is influenced by a staggering array of factors, some more important than others. Most influential are the relative sizes and numbers of the organisms as compared to a filter's restrictive pores. The physicochemical nature of the

fluid in terms of its ionic strength, pH, osmolarity, and viscosity, are other obvious influencing factors. The possibilities for the adsorptive sequestration of organisms by the filter depends upon the polarity of both their surfaces as expressed by the partial-charge induced van der Waals forces. The polymeric nature of the filter may also governs its tendency to arrest certain organisms via hydrophobic adsorptions. Additionally, susceptibility of the filter's pore sizes to alterations by contact with given solutions bears consideration. The organism size is of obvious influence. The relative ampiphilic nature of both the filter and organism surfaces can affect absorption. Common ingredients of liquid preparations such as surfactants, proteins, and charged entities such as colloids can affect the filter's ability to retain microorganisms. As previously, albeit briefly explained, filtration conditions including but not limited to temperature, viscosity, and especially differential pressure will influence filter process performance.

SUMMARY

The effective removal of microorganisms from a process stream is anything but simple. In the author's view the definition of a sterilizing filter and belief structure that has evolved around filter function are both simplistic. Filtration is all too often taken for granted in terms of both performance and outcome due to an overly optimistic belief in worst case challenge testing and a certain naivety regarding the true nature of the microbiological world. What is required is a realistic appraisal of filtration, which accepts that the actual demonstration of unequivocal "sterilization" is not currently possible.

It seems quite clear that risk mitigation in filter sterilization can only be achieved when control of bioburden is given equal or perhaps even greater weight than the successful completion of a biological filter challenge. Hence, there is obvious merit in the suggestion that control of bioburden to consistently low levels through proper process design, facility design and process control, is not just essential but mandatory. Finally, one should always bear in mind that there is firm evidence that passing the challenge test alone is no reason for a feeling of absolute safety and security. A holistic approach to the selection, validation, and use of filters in aseptic manufacturing based upon careful risk analysis and contamination control is the only way to achieve the level of confidence that production of sterile products requires.

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Filter Sizing: The Requirements and Their Attainment

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INTRODUCTION

The filtrative processing of a fluid suspension in order to separate its particulate content obviously requires an adequate expanse of effective filtration area (EFA). The volume of the preparation and its degree of loading are the determinants. Knowing the rate of filtration is important if the operation is to be designed for a batch size to meet a particular time schedule. Such may be required for compliance with regulatory needs, or out of concern for product stability, or from considerations of economy of time and/or labor, or even of convenience. The sufficiency of the filter's size to accommodate the extent and nature of the preparation's particle load needs to be known. Too small a filter will necessitate augmentation of the filtration operation in order to complete it. Mid-process interferences can be seriously disruptive, especially where sterile effluent is the goal of the filtration. Using an unnecessarily large filter would be wasteful of time and material, hold-up volume, and an indication of poor system design.

To this end the throughput of the filtration system, however defined, requires being known. Throughput will reflect the amount of particulate material to be removed, and the resulting filter-clogging and blocking characteristics under the selected filtration conditions of temperature, differential pressure, etc. This cannot be calculated. It is learned from the processing of a small volume of the preparation followed by an extrapolation to its actual volume. The more extensive the extrapolation, the less certain is its conclusion. For this reason a safety margin may be included. Where costly product is being processed, departures from the optimum filter usage can be very expensive. In addition to its EFA, the contribution of the filter's design and construction requires careful assessment. For this reason, particularly in the current production of expensive biopharmaceuticals, an upgrade in the conventional sizing practices is warranted.

In this manner the processing time is matched to the rate of flow, and the filter size is tailored to the quantity of product that is to undergo filtration.

CHOICE OF FILTER RATING

The ideal filter would ensure the required particle removal while providing as rapid a rate of flow as possible. A more open filter could be less efficient and permit the escape of

some lower size particles whose capture was desired. A too tight filter, in addition to unnecessarily restricting the flow rate, could retain finer particles whose presence is not seen as being objectionable. This could lead to an earlier and needless restriction of the throughput. The choice of the membrane filter is, therefore, worthy of deliberation.

Perhaps the best choice of the pore size rating to be used is made on the basis of a relevant experience. Where this is lacking, the selection is predicated on an estimation of the smallest particle size one wishes to remove. Experimental trial and error with different filters is then relied upon for confirmation. The pore size rating of the filter is chosen on the assumption that the size exclusion mechanism of particle retention is operative. In the case of filtration sterilizations, the 0.2/0.22-μm-rated membrane is traditionally chosen, although there is some advocacy for the use of 0.1-μm-rated filters.

The realization of filtration sterilizations is not confined to any particular pore size rating. Operational and other factors govern. The attainment of sterility employing 0.45 μm-rated membranes has been shown to be possible under appropriate conditions; chiefly low differential pressures, 14.5 psi (1 bar) in work by Tanny et al. (1979). Log reduction values of 8 were forthcoming at 29 psi (2 bar) differential pressure from each of two brands of 0.45-μm-rated membranes tested in *Brevundimonas diminuta* challenge studies (Trotter et al., 2002). The filtrative sterilization of preparations too viscous to yield practical rates of flow may be managed utilizing repetitive 0.45-μm-rated membranes. This practice is advised by FDA (1987).

It is understood that the pore size ratings are not the actual pore dimensions. Unlike the columnar pores of the track-etched membranes, the "pores" of the casting-process membranes are not integral passageways. The concept of "pore" is an artificial construct of individual spaces or vacancies within the microporous polymeric matrix that under the impetus of the partial pressure composes a pathway of least resistance to the fluid flow. Should parts of this chain of spaces becomes blocked by particle arrests, the fluid, differential pressure directed, on an ad hoc basis utilizes the available next-best adjacent spaces to continue the chain of flow (Johnston, 1992, pp. 41–5)

Like-rated membranes of different manufacture are not identical in pore size, there being no established rating standard to which pore size designations are to conform (Meltzer and Lindenblatt, 2002; Jornitz and Meltzer, 2001, Chap. 1). Moreover, the optimum filter choice is made less certain by the lack of knowledge concerning the pore size distribution, as also of the particle size distribution. Despite the absence of such pertinent data, the matching of filters to applications in the industry is usually performed successfully. The conventional technique of "trial and error" suffices for proper "pore size" choices to be made. On the basis of a long experience filter users in the pharmaceutical industry usually compensate for the differences.

RATE OF FLOW

The insertion of a filter into a fluid stream creates an impediment to flow. To overcome the resistance, a higher pressure must be imposed upstream of the filter. The difference in the pressures, ΔP, upstream and down, determines the rate of flow. For Newtonian fluids, flow rate (Q) is directly related both to the differential pressure (ΔP) and the EFA. For a constant flow rate of a clean liquid (i.e., absent particles), differential pressure and EFA bear an inverse relationship. It follows that changing one of these parameters involves alterations in one of the others. These relations are proportionate. Changes in one parameter by some percentage or multiple will necessitate a change in a second parameter by the same percentage or multiple.

Flow rate per differential pressure per EFA equals a definite value. To double the flow rate requires either doubling the ΔP *or* the EFA while keeping the other constant. To decrease the ΔP but to maintain the same flow rate demands an increase in EFA. The necessitated increase in flow rate achieved at a reduced pressure could, in a case where the particulate loading is high, result in a greater throughput by minimizing compaction of the retained matter. The desired increase in EFA can be arranged by the use of parallel filtration. Filter costs will be increased accordingly. Alternatively, the EFA could be kept unchanged along with gaining the advantages of lower differential pressures if a lower flow rate were accepted. This would incur an increase in processing time. Concerns regarding product stability may necessitate faster processing times (Priebe et al., 2003). In short, the relationship among flow rate (Q), EFA, and differential pressure (ΔP) can be utilized in filtration design to attain particular goals (Green and Meltzer, 1987).

THE FILTER AREA

In designing a filter system, a large enough EFA should be provided to permit completion of the filtration within the span of time allotted to it. Using a larger EFA than necessary needlessly incurs the wasting of filters. An insufficiency of filters is even less desirable. It may force a mid-process change-out of filters, a most disruptive action. Expeditious filtrations are usually desired. Filter blockage by retained particles is the usual cause of flow rate reduction that may prolong a filtration. However, slower flow rates motivated by reduced ΔPs may be selected deliberately for the advantages they offer. These include the avoiding of filter cake compactions, as also of the polarized particle layer in front of the filter surface (Fig. 1). Permeate-reducing compactions may limit throughputs. In addition, lower differential pressures increase the possibilities of enhancing the adsorptive retentions of smaller particles. Slower flows will in a more leisurely way bring the particles to the filter. Ultimately the same number of particles may be removed by the filter, and the final throughput will not be encumbered. However, care must be

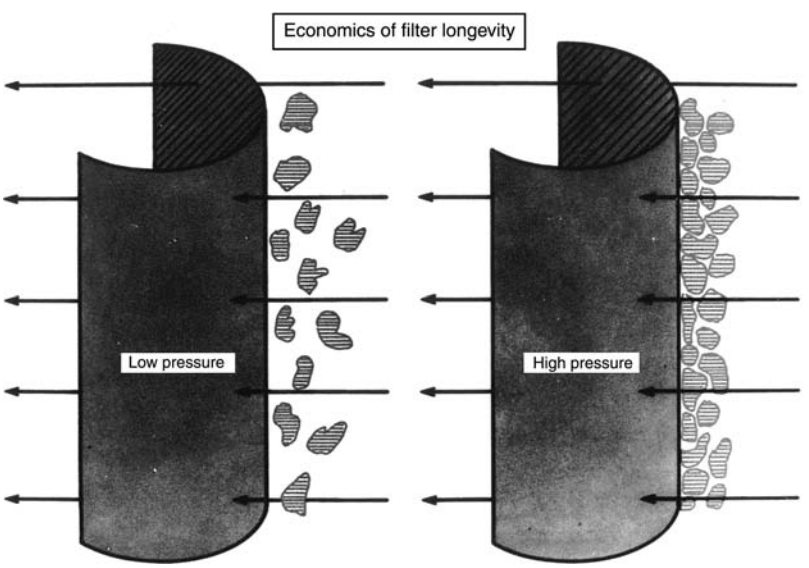


FIGURE 1 Effect of differential pressure on throughput volume. *Source*: Courtesy of Capitola Presentations.

taken that the processing of the production batch should be completed within the time allotted.

Larger expanses of membrane may have purposes other than accommodating larger particulate loads. As just stated, they may substitute for higher differential pressures in supplying more effluent volume per unit time. In this role they will yield a more diffuse particulate deposit less susceptible to permeability reductions.

VISCOSITY

Flow rate is the easiest to measure from among the filter properties of interest, namely, flow rate, throughput, and extent of particle removal. The other two properties are discerned by experimentation, but flow is, for most fluids, a product directly defined by the differential pressure, and inversely moderated by viscosity. Viscosity, in turn, is reciprocal in relation to temperature. Rates of flow can be varied by manipulating the differential pressure, and the temperature/viscosity relationship.

Flow information is normally given for water. Since the rate of flow varies inversely with viscosity the flow rates for more viscous liquid media will be reduced proportionately, and must be corrected for. Water, the standard, has an assigned numerical viscosity value of one. A liquid having a viscosity of 3 centipoise (cP) will flow one-third as fast; a liquid whose viscosity is 36 cP will flow 1/36th as rapidly; etc. The viscosity effect on rates of flow is not exact, as it ignores liquid/filter interactions which, in their extreme, manifest themselves in filter swelling and other incompatibility expressions. Fortunately, substituting other liquids for water generally minimizes these aberrations. Compatibility is also a condition of proper filter choice. Generally, liquids tend to be less viscous at elevated temperatures, and to filter more rapidly. The heating of liquids to effect more rapid filtration is usually not used, however, particularly where protein denaturation poses a threat. Some note of the liquid temperature should, nevertheless, be made.

FILTER STRUCTURE INFLUENCES

An increase in membrane area results in a proportionate increase in flow rate. Higher flows are also proportional to higher pressures, as also to lower viscosities. With regard to filter structure, fluid flows are enhanced by larger EFAs, and shorter pores, as derived from thinner membranes, and wider diameters (higher pore size ratings). Filter porosity, not too easily measured, is the ratio of void space to solid matrix that composes the filter. Cast microporous membranes are generally 70–90% porous. Thin filters are markedly proficient in rates of flow. Their manufacture by the casting technique does incur larger risks of imperfections due to their thinness. Integrity testing attests to their proper and acceptable construction.

The flow characteristics of single pores are described by the Hagen–Poiseuille Law wherein a fluid of viscosity η, with an average velocity u, of the fluid is related to the tube diameter d, and pressure drop ΔP along a length[a]

$$d^2/32 = u^*\eta^*z/\Delta P$$

[a] The flux of a membrane describes the effluent flow in terms of volume (e.g., mL) per filter area (cm^2 or $inch^2$) per unit of pressure (psi or bar), per unit of time (sec or min).

This bears on the comparison of filters for their flow qualities (Johnston, 2003). Consider a fluid of a given viscosity flowing at the same average velocity through two pores of similar length, under the impetus of an identical pressure differential. All the factors being equal except for the pore diameters, the rate of flow for the pores of the two membranes will differ as the squares of their diameters, or as the fourth power of their radii. If, however, the diameters of the pores are the same, differences in the flow velocities must derive from differences in porosity. However, this measurement of flow does not reveal either filter's retention properties.

PRESSURE UNITS

The chief motivator of liquid flow is the applied differential pressure. There is room for ambiguity because "pressure" is referred to in many different terms:

1. *Inlet pressure*: The pressure entering the inlet (upstream) side of the filter as displayed on the inlet gauge. It is also called the upstream, or line, or gauge pressure. In a closed filtration system gauges will be used upstream and down from the filter. The applied differential pressure is that of the difference between the readings of the two gauges. In a vented or open system, the downstream pressure is the ambient pressure. It equals that of the atmosphere, usually taken as being 14.5 psi (1 bar). When gauge pressure is being used, the downstream pressure is zero. The differential pressure is then equal to the line pressure. The inlet pressure is, therefore, usually expressed as gauge pressure.
2. *Outlet pressure*: The pressure exiting the downstream side of the filter. It is also called the downstream pressure.
3. *Gauge pressure*: The pressure registered on a pressure gauge at that particular line location. Since the gauges are meant to be unresponsive to ambient pressures, they register the pressure over that of the ambient. If the gauge is located upstream of the filter, and the system downstream is open to the atmosphere, the gauge pressure is read as being the indicated value, but it is actually at that level plus 14.5 psi or 1 bar. However, the gauge reading is the actual differential pressure driving the flow. It is symbolized as psig.
4. *Absolute pressure*: The pressure above a vacuum. Symbolized as psia, it would read 14.5 psi above gauge pressure.
5. *Differential pressure*: The difference between the inlet and outlet pressures. Also called ΔP, psid, transmembrane pressure (mainly used in tangential flow applications), or pressure drop. As a filtration proceeds in its particle removal function, filter cake builds and increasingly raises the resistance to flow. The difference between the up- and downstream gauges diminishes progressively; hence, the term "pressure drop." It is a measure of the filter's blockage and an indicator of how much of the membrane's capacity to accommodate particulate has been consumed, or (its reciprocal) how much yet remains available. Green and Scheer (1997) also define additional differential pressures.
6. *Maximum differential pressure*: The maximum differential pressure available for filtration after the system restrictions and the pump limitations are accounted for. Also referred to as the available differential pressure, it is typically greater than the ΔP actually used in the filtration.
7. *Maximum operating pressure*: Maximum allowable pressure to be used on a filtration system. This pressure should not be exceeded to avoid any damage to the system, for example housing.

8. *Initial differential pressure*: The differential pressure at the start of the filtration. It has significance as the clean differential pressure, unencumbered by particle depositions.
9. *Final differential pressure*: The differential pressure at the filtration's conclusion, the dirty differential pressure. It can also be a differential pressure limit specified by the filter user. Once this level has been reached the filter requires being exchanged. Preferably this pressure will not be reached before filtration end.

CONSTANT PRESSURE FILTRATIONS

Most filtrations are carried out under constant pressure conditions. It is *not* the differential pressure that is constant. The pressure referred to is a constant inlet pressure supplied by a pump whose operational setting is fixed for the duration of the filtration. The particle retention will inevitably cause a growing resistance to flow even as the inlet pressure remains constant. This results in a sequential diminution in the flow rate. The gauge pressure after the filter will indicate the growing blockage by registering progressively higher pressure readings. This leads to on-going decreases in the differential pressure since the inlet pressure remains at its constant set level. The phenomenon is referred to as "pressure drop." Its consequent reduction in flow rate continues, in accord with the developing pressure drop, to where it no longer suffices for a meaningful rate of flow. Usually, the filtration is terminated when the flow has decreased to some 20% of its starting value. However, this can be decided as being where the flow stream becomes broken, falls below a given rate, or at any other arbitrary point.

Pump Pressure Setting

In certain cases, the inlet pressure level may be limited by the pump capacity or by considerations of the sufficiency of other components of the system. Usually, the pump is set to deliver from 1 to 2 bar (14.5–29 psi), as when transferring from a formulation vessel through a sterilizing filter to a holding or storage tank. The rate of flow will decrease with time to the extent that filter blockage occurs, but the diminution in flow rate is of no consequence provided that at the end of the filtration, the batch has been put through the filter within the allotted production time. What is required of the filter, other than it display the necessary particle-trapping efficiency, is that it accommodates the required throughput volume within the time interval designated for the filtration. Parallel arrangements can be utilized to help achieve desired rates of flow.

When filling machines are involved, they may be fed from storage tanks open to atmospheric pressure, their vents protected by hydrophobic vent-filters. The filling machine pump draws the liquid to the filling needle. The filtered liquid may, in some instances however, flow directly to a filling machine without the mediation of a storage tank. In such cases, pressure regulators are used to govern the differential pressure across the filter, as well as to control the necessary pressure to the filling machine. In applications where the applied differential pressure level is not critical to filter performance (such as where filter efficiency is not paramount), the pump may be set in an inlet pressure of 3 or even 4 bar (43.5–58 psi). The filling-machine pressure regulator will then come into play to ensure proper inlet pressure to the filling machine; simultaneously defining the outlet pressure from the filter and the differential pressure across the filter.

Selection of Inlet Pressure

Trade-offs are involved in the selection of the initial differential pressure level. The higher the inlet pressure selected, the larger is the pressure drop that can result. Advantageously, this offers the largest extent of pressure loss before the filtration is terminated. Filter cartridge constructions can accommodate direct differential pressures of 72.5 psi (5 bar), back-pressures of 29 psi (2 bar), and at least 24,000 pulsations at 72.5 psi. Leahy and Sullivan (1978) found that *B. diminuta* were retained completely and equally well by a 0.22 = μm-rated membrane at 50 psi as at 5 psi (Table 1). Aicholtz (1987) confirmed that such membranes in cartridge form responded equivalently at 50 psi. However, filter efficiency, defined as percentage particle retention, as also throughput, or service life, can vary inversely with ΔP.

Higher inlet pressures will initially produce higher rates of flow, but will reduce the opportunities for adsorptive removal, and by compaction of the filter cake, as well as of the polarized particle layer sited in front of the filter (Fig. 1) will likely abbreviate the throughput, depending upon the degree of loading. The use of higher initial ΔPs reach their culminations more speedily, but are otherwise not beneficial. At lower ΔPs, the flow rates being lower, it will take longer for the retained particles to build up to the same filter blocking level. However, the total throughput volumes will be identical, except for the differences made by filter cake compaction. Thus, higher ΔPs may produce foreshortened throughputs within a briefer service life. The trade-off is between throughput volume and operational time. It may be a requirement that the batch be filtered and completed within one shift.

In conducting a filtration, the highest filter efficiency is sought, especially when filtration sterilizations are the goal. For this reason filtrations are generally not conducted at differential pressures above 29 psi (2 bar). This accords with the recommendations forthcoming from HIMA (1982). This is also the ΔP level at which the bacterial challenges performed by filter manufacturers in qualifying membranes as being of sterilizing quality are carried out; at 29 psi (2 bar). Prudently, most filtrations are conducted at constant ΔPs at that level or even lower; at 20 psi (1.3 bar).

Assessment of Remaining Filter Capacity

In addition to achieving the required particle retention level, the throughput volume is also of importance in a given filtration. Both of these qualities are reflective of the rate of flow. As stated, flow rate (Q), is related to both the differential pressure (ΔP), and to the EFA. Therefore, if a change occurs in one of these parameters, one of the others must have undergone alteration. Thus, if at a particular differential pressure a given rate of

TABLE 1 Impact of Pressure on Passage (βratio)

Filter type	Pore size (μm)	βratio		
		0.5 psid	5 psid	50 psid
GS	0.22	$>10^{10}$	$>10^{10}$	$>10^{10}$
HA	0.45	10^8	10^7	10^6
DA	0.65	10^4	10^4	10^3
AA	0.80	10^2	10^1	10^0

Source: From Jornitz and Meltzer, 2004.

flow decreases by half, it signifies that half of the EFA has been consumed, that is, 50% of the porosity is blocked by retained particulate. At this point, a doubling of the ΔP will restore the flow to its previous level. The point being made is that the loss in flow rate is also a measure of the remaining EFA. If operational alterations are to be instituted for whatever reasons, this EFA value should serve as the preferred point of departure.

Termination Point

Once the maximum ΔP has been decided upon and the pump has been set accordingly, the flow that results is inevitably fixed. The maximum differential pressure, however defined, having been utilized from the very beginning, there remains no margin for increase. Inevitably the flow will progressively diminish as the filter blocks. The inlet pressure setting remains unchanged. Each halving of the flow signals a 50% loss of filter capacity in terms of EPA. After the second halving of the initial flow rate, the filter capacity is down to 25%; and so on. The slope of the curve describing the accumulating throughput volume, or EFA reduction as a function of the pressure drop increases at a steadily diminishing rate, a geometric progression, that becomes asymptotic at about 80%.

Figure 2 illustrates this law of diminishing returns. The added throughput volume gained after 80% or so of the filter capacity has been utilized is generally not judged worth the time required to secure it. To optimize the filter economics, some threefold doubling of the initial ΔP is allowed for. Thus, one-half of 29 psi (2 bar) is 14.5 psi; one-half of which is 7.25 psi; which then halves to 3.62 psi. At this point 87.5% of the filter capacity has been consumed. To achieve the point of diminishing returns, the filtration should be terminated when the pressure differential reaches 5 or 6 psi (0.4 bar).

In sum, the rate of flow in constant-pressure filtrations starts at a maximum, and decreases in accordance with the filter-blocking propensities of the liquid suspension. Generally, the filtration is ended when the rate of flow has decreased to some 20% of its original value, or to the point where the filtrate becomes a broken stream, or in accordance with some other user preference.

If the differential pressure decreases to an unproductive level due to filter blockage caused by particle accretion, and a constant or at least a continuing flow is desired, either the EFA or the upstream pressure must be increased proportionately. The latter however, changes a constant pressure filtration into a constant volume filtration.

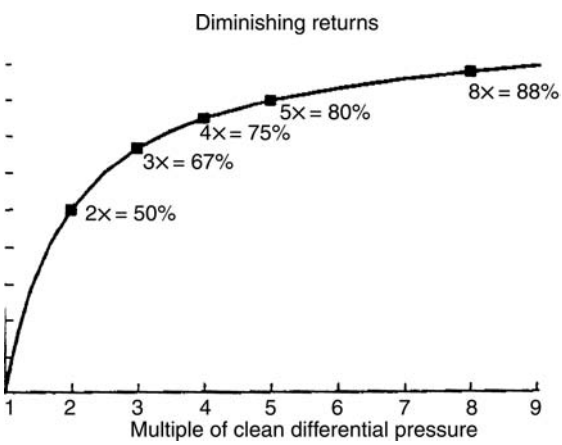


FIGURE 2 Law of diminishing returns. *Source*: From Green and Meltzer, 1987.

CONSTANT VOLUME OR FLOW FILTRATIONS

The operational conveniences of constant-pressure filtrations do not always suit. Thus, in filtering serum, where throughput rather than rate of flow is important, low-initial inlet pressures are used. As the rate of flow decreases to an unacceptable level, the impediment to flow is overcome by *moderately* increasing the inlet pressure. Filter clogging is being avoided. Once incurred, it may be impossible to reverse or remove. By means of such progressive incremental boosting of inlet-pressure, the differential pressure is increased, and the flow rate is restored. Thus, filtrations are maintained at realistic levels while enabling an optimum throughput to be achieved, albeit slowly.

Constant Volume Serum Filtrations

In one instance, the use of an initial 1.8–2.7 bar (26–39 psi) inlet pressure caused an almost immediate flow cessation in a serum filtration. Lowering the initial inlet pressure to 0.5 bar resulted in flow resumption. Usually, however, in serum filtrations, once cessation caused by excessive pressure has ensued, flow will not be revived by a lowering of pressure.

Serum filtrations require time and large filter areas. Constant-volume filtrations are usually employed because, although the rates of flow are of secondary importance, the maintenance of a low but constant rate of flow serves to control and avoid the impress of harmful higher inlet pressure levels on the filtration. The inlet pressure is varied periodically only enough to overcome the increasing resistance to flow caused by the progressive blockage of the filter, as signaled by decreasing flow.

In the constant-volume mode of filtration, then, one seeks to maintain a steady rate of flow by manipulating the inlet pressure as a means of controlling the applied differential pressure to minimize the flow decay occasioned by progressive filter blockage.

It cannot be overemphasized that proper filter system design requires the operational assessments of flow decay studies. Where these are not possible, approximations can be forthcoming from data presented in filter manufacturers' catalogs.

Parallel Arrangements

If, at the peak differential pressure, still yielding satisfactory particle/organisms retentions, the rate of flow is insufficient, the filter efficiency should not be put at risk by boosting ΔP. Rather, additional filter area should be utilized in a parallel disposition to increase the flow rate and the throughput level.

As is illustrated in Figure 3, the purpose of a parallel filter arrangement is to enhance the rate of flow and the throughput by use of more extensive EFA. In effect, the particulate deposition is dispersed over a larger area. Each filter confronts less of the total particle load. Blockage of the individual filter is thereby minimized. The realization of extending the throughput volume by managing the wider dispersal of the retained particulate matter depends upon the degree of loading.

In pharmaceutical settings such constructions providing the additional EFA in the form of additional filters being fed from the same liquid source and exiting to a common pool are called parallel arrangements. They are especially useful for filtering highly loaded fluids. The additional cartridges may be utilized in their separate housings.

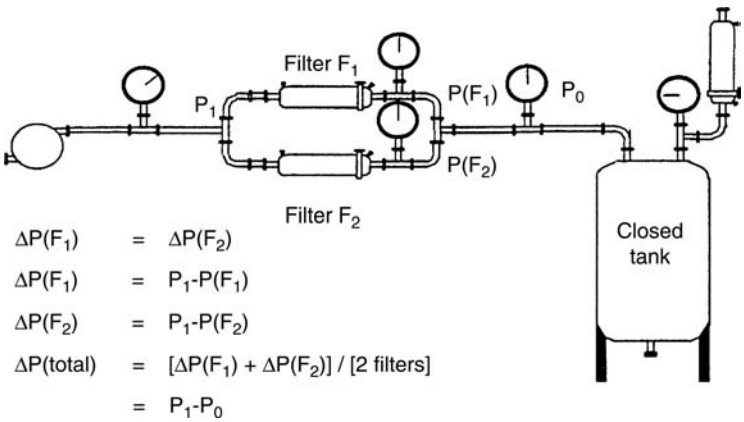


FIGURE 3 Parallel filtration. *Source*: From Green and Scheer, 1998.

However, the term is not applied to multihoused filters although they manage the same flow process to the same effect (Fig. 4). Essentially, a greater area of membrane is supplied to prevent (or slow) filter blockage by particle accretion.

Each filter operates at full differential pressure to give a total rate of flow whose value is the appropriate multiple of one individual unit. The pressure drop across each of the individual parallel flow paths is equal. The pressure drop is not increased by the added membranes, because the number of parallel filtration paths does not increase the overall



FIGURE 4 Multihoused filters. *Source*: From Jornitz, 2005.

applied differential pressure of the system. Indeed, the more parallel paths, the lower the ΔP necessary to provide a given total rate of flow and throughput volume. The parallel flow arrangement can, therefore, be used to increase the rate of flow of a filtration at a given differential pressure.

Parallel filtration, while maintaining a constant rate of flow from a filtration, can be used to reduce the applied differential pressure over a given filter area, thus increasing both filter efficiency and longevity. For example, consider a filtration whose purpose is the sterilization of a batch of small-volume parenteral preparation on its way from the batch preparation vessel to a dwell tank prior to the filling machine. The filter indicated will, therefore, be of a 0.2-μm pore-size rating, requiring autoclave sterilization and integrity testing, such as by the bubble-point procedure. Ambient temperature will prevail; the liquid viscosity is that of water, 1 cP. Assume that a product flow of not less than 10 gal/min (37.9 l/min) will be required, and the initial inlet pressure used is to be set at a constant 1.4 bar (20 psi) differential, the initial drop being from 20 psig (1.4 bar), to ambient pressure.

Of the many filters available for this purpose from the several manufacturers, one could choose a cartridge that has a flow rate in the 10 inch or 25.4 cm length of some 8 gal or 30 l per min at 1.4 bar or 20 psi differential pressure. Alternatively, one can read the flow rate per 1 psi differential from the filter manufacturer's catalog, namely, 0.4 gal/min. (1.5 l/min) and multiply by 20 psi (1.4 bar) to yield 8 gal/min (30.4 l/min) per 10 inch (25.4 cm) cartridge. The viscosity of this small-volume parenteral preparation being 1 cp, the same as that of water, no correction for viscosity is required. The flow through a 10-inch cartridge will be 8 gal/min (30.4 l/min). However, the desired flow is 10 gal/min (37.8 l/min) more filter area than that provided by one 10" cartridge is required.

The necessary filter area can be provided best and most simply by the use of one 20 inch, 50.8 cm cartridge in a single holder. If such a 20 inch length cartridge were not available, two 10 inch cartridges could be mounted in a parallel arrangement, as shown in Figure 3.

Each 10 inch cartridge operates at the full differential pressure of 20 psi (1.4 bar) and independently delivers 8 gal/min (30.4 l/min of product (except as this quantity becomes progressively decreased by ensuing filter blockage).

A general formula applies:

$$\frac{\text{Flow required}}{\text{Flow per 10 inch (25.4 cm cartridge at 1 psi or 0.7 kg/cm}^2\text{ or 0.7 kg/cm}^2\text{) actual differential needed}} = \begin{matrix}\text{Number of 10 inch}\\ \text{(25.4 cm cartridges}\\ \text{psi or kg/cm}^2\text{)}\\ \text{(liquid viscosity)}\end{matrix}$$

Parallel flows can be used to increase the total rate of flow and the total throughput volume over a given time at a constant pressure. They also permit the attainment of a given volume of filtrate at a lower different pressure. Filtrations performed at lower differential pressures tend to increase the filtration efficiency in addition to prolonging the filter system's service life.

Series Arrangements

Filter dispositions in which fluid flows from one filter through another is termed a serial filtration (Fig. 5). It provides the prefilter/final filter combination with enhanced flow

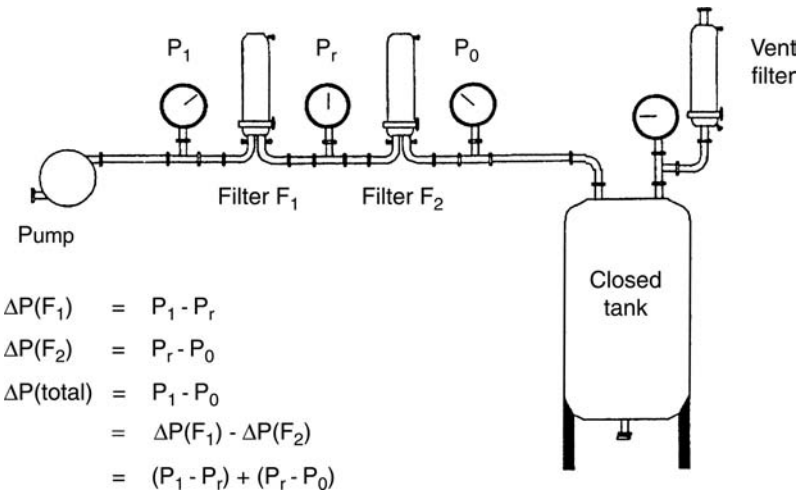


FIGURE 5 Series filtration. *Source*: From Green and Scheer, 1998.

rates. In effect, the prefilter increases the EFA to the extent that is distributes the particulate load between two or more filters. This prolongs the service life of the filtration unit as measured by its throughput. The rate of flow decay is diminished. As a result the rate of filtration may be improved. The extent of improvement depends upon the preparation's total suspended solids (TSS) load. The efficiency of the final filter is thereby increased. Correctly sized, the serial arrangement avoids the troublesome change-out of filters during the filtration process.

Alternatively, the serial filtration may be intended to ensure against imperfect particle retention on the part of the final filter. In this type of application the filter combination is a special case of repetitive filtrations. Prefiltration is a frequent practice in Europe. It extends the service life of the filter couple, increases the throughput, and minimizes the interference with the flow rate by the retained particles.

The serial coupling of the filters, for whatever purpose, inevitably increases the pressure drop across the filter train. The pressure drop is the chief penalty of the series arrangement. It increases the pumping costs, and may require the expense of additional housings. Again, depending upon the TSS, the improvements in the rates of flow occasioned by the distribution of the load over a larger filter area may be negated by the lower differential pressures that result from the two filters being in a serial alignment.

DOUBLE LAYER FILTERS

Double layer filters are a special case of the repetitive filter arrangement wherein the two membranes are included within a single cartridge. Two types of double layer filter constructions are available. The two different constructions are intended for different purposes. Both are, however, in the form of serial filters.

The heterogeneous type consists of layers of membrane of dissimilar pore size ratings. An example of a heterogeneous cartridge construction would be a 0.45-μm-rated membrane upstream from a 0.2/0.22-μm-rated final filter (Fig. 6). The homogeneous type is composed of two membranes of the same pore size, for example, both 0.2-μm-rated (Fig. 7).

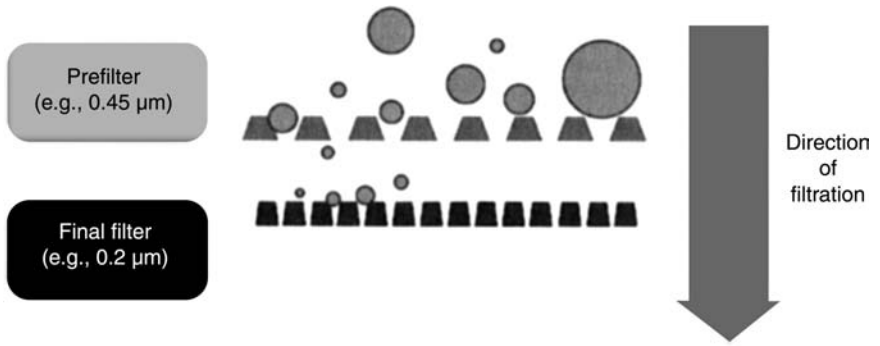


FIGURE 6 Heterogeneous double layer. *Source*: From Jornitz and Meltzer, 2001.

Heterogeneous Double Layer Filters

An example of a heterogeneous cartidge construction would be a 0.45-µm-rated membrane upstream from a 0.2/0.22-µm-rated final filter. The more open filter, in its position upstream of the tighter, serves in the protective role of prefilter; accepting a portion of the particulate load, and thereby prolonging the service life of the final downstream membrane. The rate of flow is slowed by the longer overall flow path of the double construction. It is reduced from that of either single membrane. However, the prefilter effect is to prolong the throughput by dispersing the particulate burden between two filters. Depending upon the numbers, sizes, and shapes of the particles, the effect should be an increase of the throughput volume.

By way of automated integrity testing machines that avoid invasions of the filtration train downstream of the final filter, the integrity testing of a filter can be performed without endangering the asepsis of the system. When repetitive filters are used, each in its own housing, each can be integrity tested separately. When in the same holder, neither filter can be tested without invading the space separating them unless some very unusual arrangements are devised. This is one disadvantage of relying on repetitive filters.

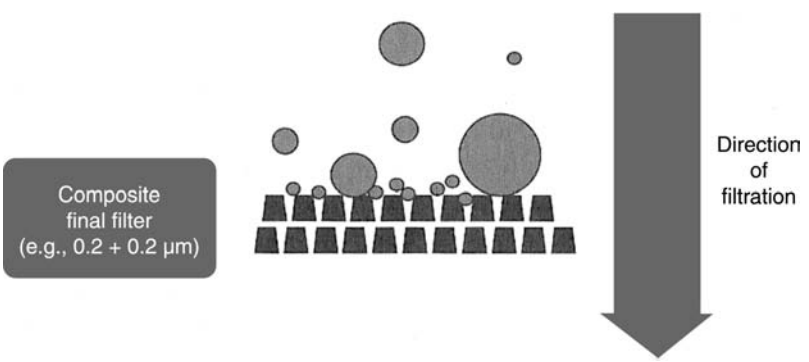


FIGURE 7 Homogeneous double layer. *Source*: From Jornitz and Meltzer, 2001.

Homogeneous Double Layer Filters

The homogeneous double constructions contain two membranes of like pore size ratings. This double layer construction reduces the probability of particle passage. The intention is to ensure that even if an inappropriate or flawed membrane were somehow included, the second filter would serve as a safeguard. Likewise when single membranes cannot be cast to thicknesses sufficient to retain the target organisms, they too are used in double constructions. The second filter serves as an abettor to the first, reinforcing its intended action of particle retention.

The clean rate of flow through double filter constructions is less than that through single membrane layers. This is due to the pressure drop that results from the overall pore lengths being longer. The clean rate of flow through homogeneous arrangements is less than through heterogeneous double filters because in the former combination both membranes have the tighter restrictions of the final filter.

Repetitive Filters

The pore is modeled as being a convoluted, irregular capillary. Because of its tortuosity, it is necessarily longer than the thickness of the filter. Its restrictive dimensions may occur anywhere along its length. This assumption explains certain views regarding repetitive filters. It would be expected that lengthening a pore might proportionally increase the pressure drop, i.e., decrease the differential pressure across its span. This should reduce its flow rate, promote its adsorptive particle-trapping abilities, but probably not alter its throughput. The actual situation wherein membranes are superimposed in intimate contact on one another in effect does prolong the pore length.

This congruent positioning of the filter layers will cause a narrowing of the pore size distribution. Fewer large size pores will result because such pores in the one filter will likely superimpose those of smaller ratings in the other since there are many more of the smaller. The result will be a diminution in the overall pore size, and in their distribution. This should assist sieve retentions. Moreover, the pore paths are doubled in their lengths, as are also their surfaces. This encourages adsorptive sequestrations as particles being carried through the pore passages more frequently undergo Brownian type collisions likely to cause their impactions with the pore walls. The filter efficiency is enhanced thereby.

When use is made of two membranes that are separated from one another, a different result is obtained. Between the two membranes there exists a space wherein the fluid exiting the first membrane forms a pool from which it is hydrodynamically directed to the larger pores of the second membrane. The hydrodynamic flows preferentially convey particles to the more open pores. This detracts from the narrowing of the pore size distribution's doubling of the single filter's pore length (Reti et al., 1979) (Fig. 8).

To repeat, the log reduction values (LRVs) of individual filters are not additive in their combinations (Trotter et al., 2000; Reti et al., 1979). The exact sum depends upon the probabilities of given size organisms meeting an appropriately sized pore within the pore size distribution. Whether by encounter with a larger pore, or with a flaw, the likelihood is that the organisms penetrating the upstream filter will be the smaller of the organism present in the feed waters. This will decrease the size distribution of the organisms downstream of the first filter in the direction of overall smaller sizes. This, in turn, makes more likely a lower LRV for the downstream filter.

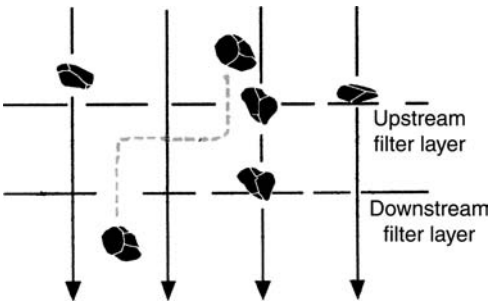


FIGURE 8 Effect of repetitive layer on particles retention and integrity testing. *Source*: From Priebe, Jornitz, and Meltzer, 2003.

Redundant Filtration

This term is often used to designate a repetitive or two filter arrangement wherein the two filters, each in its own cartridge, are housed separately. The term "redundant" often conveys a pejorative cast to the usage as being wasteful or unnecessary. Its intended meaning in filtration is to imply "enough and to spare." The first filter, as is common to repetitive filters, is expected to fulfill the retention requirement. The second filter serves as insurance. It is a safeguard that by its very presence is worth the cost of the greater certainty it supplies. As already observed, the LRVs of repetitive filters are not twice the value of a separate filter.

Redundant filtration incurs the cost of two housings, but the separate housings permit the integrity testing of each filter, albeit the risks to asepsis inherent in prefiltration integrity testing still apply. Within a single housing the upstream testing of the downstream filter is manageable, but will require the isolation of the upstream filter.

REGULATORY RECOMMENDATIONS

As regards the EU and FDA, both the guideline CPMP, April 1996, and EC Annex 1, as well as the FDA's Aseptic Guide (2002) recommend the use of redundant 0.2/0.22-μm-rated membranes. FDA's new aseptic guideline states, "use of redundant filtration should be considered in many cases." The placement of the filters is to be "as close as possible" (or practical) to the filling needles. The usual location is indeed just before the filling needles or before the reservoir that feeds them. In practice the "recommendations" are enforced as if they were law. In Europe, this usage is becoming the common practice, whereby the difference between redundant and serial filtration has to be regarded. Redundant is defined as two filter of the same pore being connected. Whereas, serial filtration means, commonly, a piece of equipment, usually a tank, is connected by two filters of the same pore size.

The recommendation is not based on any known survey or experimental data, nor have the regulatory authorities explained their reasoning. Those responsible for the public safety are often obliged to make decisions when adequate supporting data are not yet available. Over-design is a normal response to uncertainties. By way of automated integrity testing machines, that avoid invasions of the filtration train downstream of the final filter, the integrity testing of a filter can be performed without endangering the asepsis of the system. When repetitive filters are used, each in its own housing each can be integrity tested separately. When in the same holder, neither filter can be tested without invading the space separating them unless some very unusual arrangements are devised. This is one disadvantage of relying on repetitive filters.

Prefilter Action

What is required of a final filter is that it reliably retains the particles it is proposed to remove from the fluid stream being processed. In pursuit of this reliability, membrane filters, characterized by their narrow pore size distribution, are usually selected for critical operations. The pore-size designation is made on the basis of the presumed particle size. This is seldom known with certainty, but experience with the application can serve as a guide. Membranes are available in smaller pore-size ratings than are depth-type filters; their ratings are more dependably defined; and, as said, their pore-size distributions are narrower as a consequence of their technologies of manufacture. As a result, their particle retentions are more thorough. However, in retaining higher particle loads they may sooner undergo blockage, leading to a troublesome need for mid-process filter replacements. Alternatively, more extensive filter area can be used at the outset. Also, a prefilter(s) may be employed. Its function is the acceptance of part of the particle load, thereby sparing the final filter that burden, and prolonging its service life (Fig. 9). Depth type filters are usually used for prefiltrations. However, membranes of higher pore size ratings may serve as prefilters to final filters of finer porosities. In such cases it were best, however, that the liquid not be highly loaded, or that more extensive EFA be used to forestall premature filter blockage (Trotter et al., 2002; Jornitz and Meltzer, 2001, Chap. 4).

Prefilters are not intended to be completely retentive (if they were, they would by definition be final filters.) Prefilters are designed to accommodate only a portion of the particulate load, permitting the remainder to impinge upon the final filter. Figure 10 illustrates the retention efficiency for a series of depth prefilters of different pore size ratings. In the process, the life of the final filter is prolonged by the intercession of the prefilter, whose own service life is not unacceptably abbreviated thereby. Overall, the service life of the prefilter(s)/final filter assembly is extended to the point where the rate of fluid flow and its throughput volume meet practical process requirements.

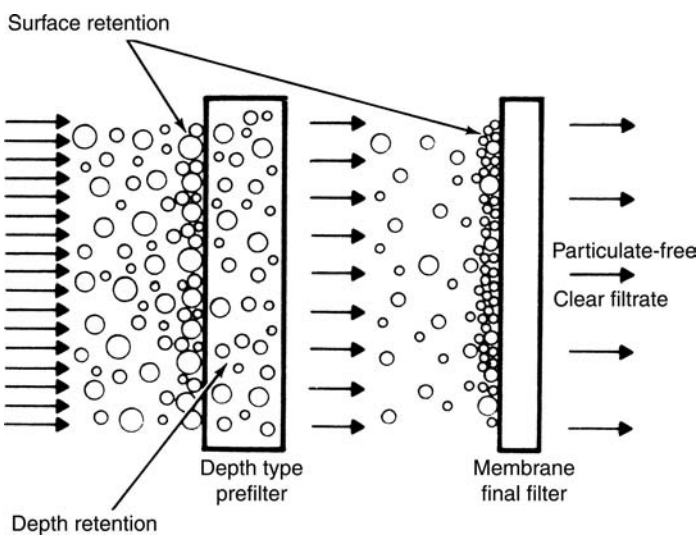


FIGURE 9 Prefilter-final filter combination. *Source*: From Lukaszewicz, Johnston, and Meltzer, 1981.

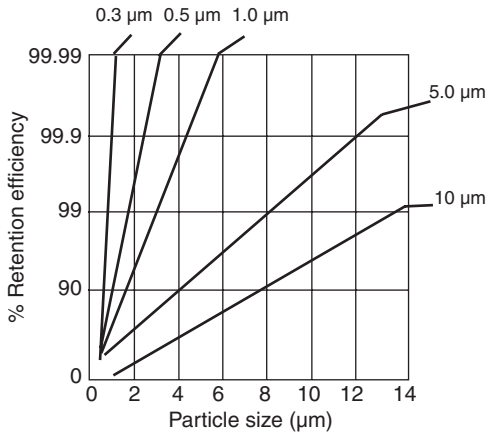


FIGURE 10 Retention efficiency curves for a series depth prefilter catridges. *Source*: From Jornitz, 1995.

PREFILTERS AND FINAL FILTER EFFICIENCY

Not only does the use of prefilters serve the function of increasing the EFA of the system, but it may increase the filter efficiency as well. Filter efficiency may be defined as the percentage of influent particles that are retained by the filter. When the sieve-retention mechanism of particle arrest is not the sole means of organism capture, the filter efficiency may be an inverse function of the organism challenge level. It depends on the particle size distribution. The two filter combination is more likely to arrest particles small enough to penetrate one filter. In effect, the longer pore length supplied by the filter pair provides greater opportunity for a smaller particle being carried within the passageway to encounter the pore wall in response to Brownian motion. An adsorptive interaction between particle and pore surface could result.

By accepting a portion of the load, the prefilter attenuates the rate of flow decay of the final filter; thereby increasing the throughput. In reducing the challenge level of larger particles to the final filter, it may leave available the smaller pores of that filter that might otherwise be preempted. This could increase the filter's efficiency for adsorptive captures.

Prefilter Selection by Flow Decay

A filter's clean rate of flow, that is, obtained by the use of a clean fluid, mirrors such fluid characteristics as viscosity, temperature, etc., and its degree of interaction, if any, with the filter. The rates of flow of fluids containing particles reflect the progressive pore-blocking caused by the filter's retention of the particles, especially as aggravated by the differential pressure's compaction of the filter cake they form. This (dirty) rate of flow is the rate of flow decay. The total *throughput* volume given by a filter will chiefly depend upon the particle-size/pore-size relationship. The filtration conditions, the differential pressure in particular, will strongly affect the results. The determinants are the filter's EFA; its thinness, its total porosity (the number and sizes of its pores); and the number and sizes of the particles it retains, which is to say upon the TSS content and its concentration. It may be that a given filter/liquid combination may not yield the required throughput, or that the flow rate may not produce it in a timely fashion. In such instances reliance may be made

upon the use of prefilters to correct the situation. A proper prefilter selection can be made by the use of flow decay measurements.

Flow decay measurements, the plotting of flow (throughput to time *t*) as a function of time, will reveal the flow-limiting effects of the particle load. This is first performed for the proposed processing filter. From this will be learned whether the selected (final) filter will yield adequate flow rates, retentions, and throughputs.

The same type determination following the use of a prefilter, will illustrate the effect of the prefilter on the rate of decay. Comparison of both flow decay curves will show the ameliorating effect of employing the prefilter. What is desired of a prefilter is that it should so modify the flow decay curve as to enable acceptable throughputs over an acceptable interval of time (Fig. 11). The assaying of different prefilters allows comparisons to be made regarding their relative efficiencies. If necessary, more than one prefilter can be used in concert with the final membrane to compose the filter assembly. Illustrated in Figure 12 is the optimization by prefilters of a 1.25% tripticase soy broth preparation.

USING FILTER MODELS

These flow decay studies are traditionally performed using volumes of the drug preparation small enough to be assayed using 47-mm flat disc filters. The results obtained are then extrapolated to the membrane EFA that will be needed for the drug volume being processed. It is the employment of just such flow decay investigations that can be used to estimate the best filter combinations.

Obviously, flow decay models as apt as possible should be used to obtain data whose extension will lead to reliable filter scale-ups. It is convenient and economical, both in terms of effort and material costs, to use the 47-mm flat discs, for "indicator trial" measurements of flow rates and throughputs. However, it is too often assumed that in the sizing of filters an extrapolation can be made from small flat discs to pleated filter cartridges large enough to handle production requirements. Meaningful scale-up or sizing to meet the needs of the process that will rely on pleated cartridges can only be done with pleated filter devices. Flow cannot adequately be measured and extrapolated from flat

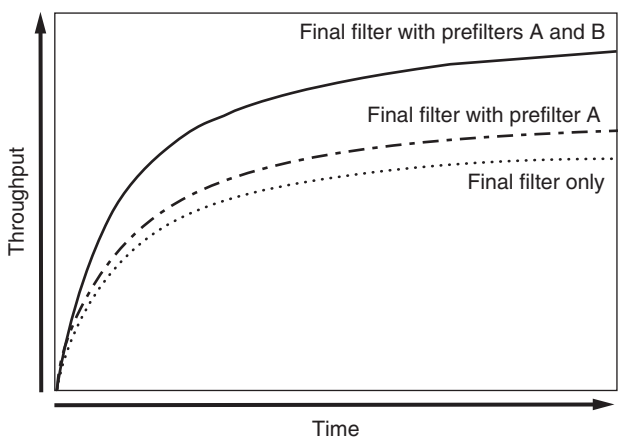


FIGURE 11 Extending filter life with prefiltration. *Source*: From Lukaszewicz, Johnston, and Meltzer, 1981.

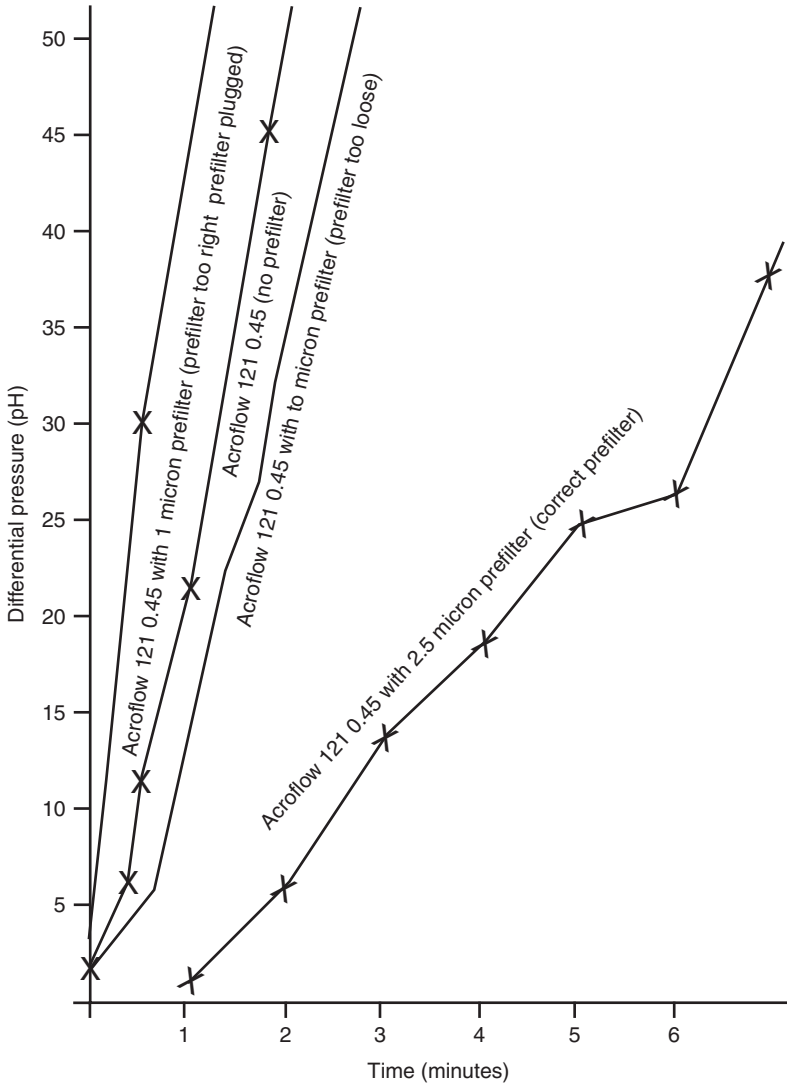


FIGURE 12 Throughput of trypticase soy broth (1.25%) effects of prefiltration. *Source*: From Lukaszewicz, Johnston, and Meltzer, 1981.

stock to pleated membranes due to influences inherent in the pleating process. The measurement of the EFAs of flat disc filters is uncomplicated. That of pleated filters is appreciably more complex. The flow rate scale-up using 47-mm flat discs reflects only the influences of the membrane thickness and its porosity. The modifications in EFA resulting from pleat-pack constructions must not be neglected. These derive from the flow-attenuating influences of the cartridge's support and drainage layers. To this must be added the reduction in flow caused by the occurrence of pleat compaction. The utmost caution has to be exercised when filter choices are made by evaluating flow rates using 47-mm flat disc filters. The flow data will not extrapolate well from flat stock to pleated filter cartridges.

A more responsible action is to follow the "indicator trials" using 47-mm discs with "verification trials" wherein the total throughput and flow rate measurements are

obtained by the use of pleated devices. Small pleated membrane units, such as capsules or mini-cartridges are available for this purpose. These "verification trials" are needed to more quantitatively confirm the results gained from using 47-mm disc filters. It is from the "verification trials" with small pleated devices that extrapolations to pleated cartridges can be extended to full productions.

Where expensive preparations are being dealt with, as in the biopharmaceutical industry, an even more accurate scale-up operation should be based on full scale "assurance trials." These should be undertaken before the full production run is ventured. If necessary, a full 10-inch cartridge should be employed as a model. If the drug preparation is too expensive for such trials, water, or an aqueous fluid compounded to a viscosity similar to that of the preparation should be relied upon.

It should be pointed out in passing that the wrong perceptions derived from inappropriate tests may prove expensively misleading. Failures to properly assess correct choices in the scale-up process may result in the rejection of filters that could perform appropriately in production runs.

PLEATING EFFECT ON THROUGHPUT AND FLOW DECAY

Production runs will probably require pleated cartridges to supply the larger EFA that will be needed. The projection of flow decay data from flat discs, as said, is *inappropriate* in determining the required EFA when it is to be secured as pleated cartridges. Cartridges are more complex in their construction than are simple flat discs. The pleating will indeed furnish the added EFA that is its purpose. However, the flow dynamics, the pleat architecture itself, the number of pleats and their heights, the nature of the support and drainage layers, and possibly more subtle performance parameters may detract from the full potential offered by the pleating. Figure 13 illustrates some of the different pleated filter designs that are available, conceivably for different effects. Densification of the filter-pack may conceivably contribute to a greater retention of particles. Were this to lead to an earlier filter blockage, the throughput would be negatively affected. In essence it would be as if less EFA than had been expected were at hand. This may be one reason why conclusions forthcoming from flow decay studies, while accepted, are paired with the caveat of a generous safety margin (Jornitz et al., 2004). Therefore, following the preliminary 47-mm disc "indicator trials" identification of the proper filter combination necessitates follow-ups with either "verification" or "assurance" trials employing pleated filter devices.

The practice of sizing the production filter by an ultimate reliance on 47-mm discs is still experienced within the industry. It should not be. It may suffice where the filtration deals with relatively inexpensive products whose mishandling occasioned by unsuitable sizing operations will be relatively inconsequential. It is surely inappropriate in the biopharmaceutical industry where the products are of a significant value and where their losses will be costly. The 47-mm disc usage can lead to estimates of flow rates and throughput values. However, only pleated elements can reliably be used to predict the performance of pleated cartridges. Required, at least, is the use of small scale pleated devices. Such are available as capsules or mini-cartridges.

The EFA of 47-mm Discs

The EFAs of a 47-mm flat disc filter is 17.4 cm^2 in area. Use is made of this number in system sizing work wherein this expanse of filter surface yielding a given amount of throughput is employed to determine by way of ratios what expanse of filter area would

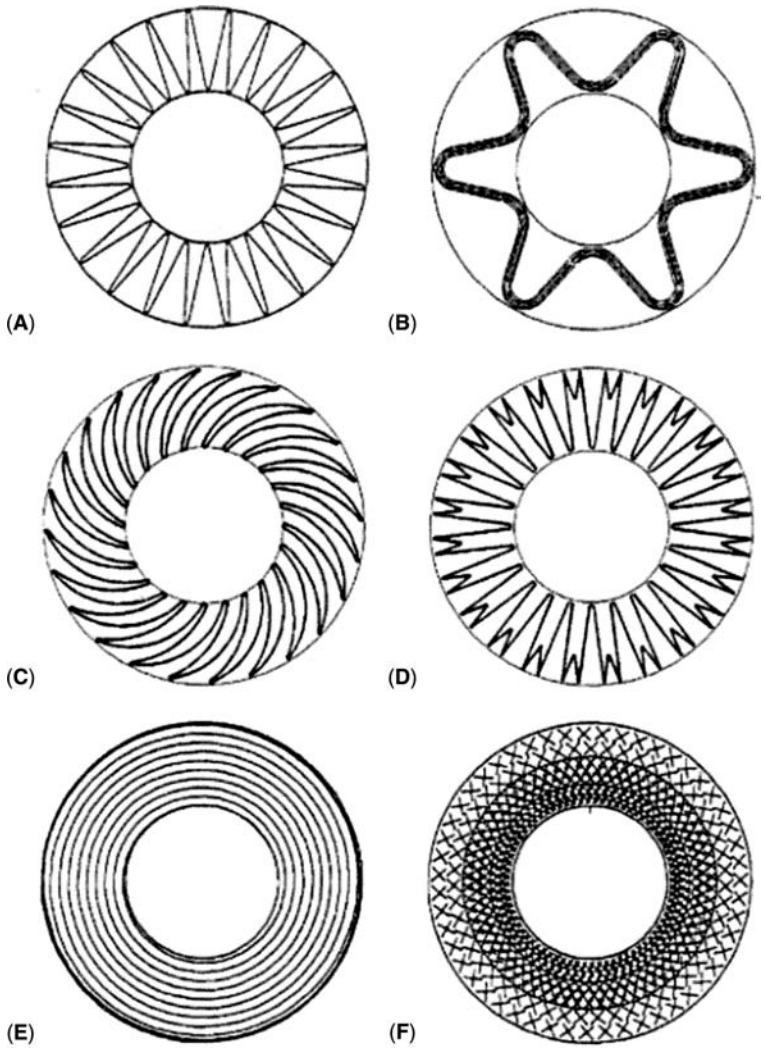


FIGURE 13 Pleated filter designs. *Source*: From Soelkner and Rupp, 1998.

be needed to produce a specified volume of product in actual productions. The use of the 47-mm discs for this purpose is widespread although the operational details may differ among its users, there being no standardized operational steps.

In the classic usage of 47-mm disc filters, a membrane is removed from its package, and is inserted into a stainless steel holder wherein it is held in position, sealed by the compressive action of O-rings. The EFA of the 47-mm disc is 17.4 cm^2. However, in the holder the O-rings preempt some of the disc's peripheral space. The actual effective filter area of such an assembly available to the liquids being treated was identified by filtering a staining solution of acridine yellow. The stained area measured 41 mm in diameter. Its area was calculated (π/4 d2) to be 13.2 cm^2. This accords with the defined meaning of EFA.

It is not known what number value the many users of 47-mm disc filters may individually employ in their sizing protocols. The measurement of actual filter EFAs by acridine staining seems not to be a widely recognized procedure. It is little discussed or

published. Certainly its application to the use of 47 mm in the present context is hardly universal. Yet, its usage discloses the disc's EFA with great exactness.

If the EFA value used for the 47-mm flat filter is that of the full 47 mm dimension, while its actual use-area in the application is 13.2 cm^2, then the extrapolations made would lead to expectations of larger flows than will actually be realized. The extrapolation will fall short of the actual need. This may compel a mid-operational addition of more filter area.

The absence of industry standards in this instance is exacerbated by the appearance on the market of a "47-mm disc" pre-sealed in a disposable holder. The staining technique reveals its diameter to be 48 mm. This is equivalent to an EFA of 18.4 cm^2. If its diameter is exactly 47 mm, then its EFA would be 17.4 cm^2. This compares with the replaceable individual inserts' EFA of 13.2 cm^2.

The opportunities for inaccuracies in sizing decisions based on the use of 47 mm disc filters argues for follow-up "verification testing" with larger filter units. This could be the prelude to the infinitely more reliable "assurance testing" wherein full scale modeling would be relied upon.

INHOMOGENEITY CONSEQUENCES

Some degree of filter inhomogeneity, in terms of porosity, could eventuate from membrane pore size distributions. Its manifestation would bear some inverse relationship to the EFA of flat disc membranes, but only after a certain low level of EFA is attained. The fewer larger pores of the pore size distribution may not be numerous enough to be part of every small area of membrane that may be cut from the membrane role. Therefore, as previously stated, the homogeneity would asserts itself only in membranes of low EFA; for example, flat discs of 47, or so, mm size.

Such inhomogeneity would be of very limited significance in pharmaceutical processing operations where the large EFAs of pleated cartridges are utilized. However, it could perhaps have some negative consequences where the sizing of filters is performed by flow decay measurements on 47-mm filters. The probability of such a situation would be minimized by trial replications. This possibility argues for the use of test filters larger than the 47-mm discs customarily employed. In any case, the use of membranes of larger EFAs would involve the measurement of larger volumes of test liquids. The data, consisting of larger integers, should result in more reliable extrapolations.

Flow Decay Procedure

Number of Housings

Tests are sometimes conducted by placing simultaneously as many different filter/prefilter discs into a single holder as will fit while still providing adequate sealing and freedom from interference. This method is simpler than using individual holders for each filter, but it will not reflect as much useful data. By using a single disc holder, the flow decay determination will, by experimental design, yield equivalent areas for the prefilters and final filter. This limits the utility of the flow decay to providing information on the proper pore size and material selection. It will not provide any information on the ratio of prefilter/final filter areas. The suggested method is to use a separate filter holder for each simulated filter, to obtain data on the individual units, collect the effluent, and to filter the

solution through the next filter unit in series. Using this approach will generate both the next filter in series. Using this approach will generate data both on proper pore-size rating selections and on relative prefilter/final filter areas.

However, when a serial or double-layer-design cartridge filter is being considered, this construction must be reflected in the flow decay test. In this case, the multiple layers should be placed in the same disc holder, since this will automatically yield the equivalent areas inherent in the serial cartridge design.

Two 47-mm filter holders are connected in series. One end of this arrangement is connected to the source of the fluid whose filtration is being tested. The effluent end of the filter combination leads to a collecting vessel with volume calibrations so that filtered liquid volumes can be read. A 2-l graduated cylinder serves nicely where water samples are being tested. For fluids such as serum, a 100- or 250-ml graduated cylinder suffices. The applied pressures should be measurable. A stopwatch or timer is also required. The flow decay should be monitored by recording data at pre-selected time intervals. The intervals may be modified as the decay progresses. It is suggested that either flow rate versus time (in a constant differential pressure system) or differential pressure versus time (in a constant flow rate system) be recorded.

Recently these filterability test rigs became automated, that is, the filtered volume is measured with a balance, the differential pressure is evaluated with pressure transducers and the data are processed with a data acquisition system. A software program calculates a certain square foot predictor and plots the graphs of differential pressure, flow, and volume. These systems are extremely accurate filterability devices, and work very well also in regard to retaining and storing the evaluated data within a computer system.

The liquid is passed through a filter placed in the downstream filter holder. For the moment, the upstream holder remains without a filter. The filter used is of the degree of fineness required for the ultimate treatment of the liquid. Flow is continued until the point of filter insufficiency or of shutdown is reached. During the flow, measurement of the time interval to pass a given small volume of the liquid is frequently made. Alternatively, the volume of flow per fixed time interval is measured. Either way, the ongoing rate of flow is frequently checked and plotted. A slowdown in rate signals the advent of filter insufficiency. This endpoint is most reliable when the applied pressure is kept constant. The total volume that is filtered to the point of shutdown is recorded, as is also the corresponding length of time.

The point of filter insufficiency is reached when the rate of liquid flow, its volume per unit time, falls below a certain desired amount. Usually a decrease in the rate of flow to about 20% of its initial value signals an endpoint. In different contexts other indicators may be used as appropriate. This cutoff point is regarded as the point of diminishing returns.

The procedure is then repeated, but this time with a more open filter in the upstream holder along with the ultimate fine filter in the downstream holder. The upstream filter serves as a prefilter to the other. The efficacy of its prefiltering role is indicated by how much added volume of liquid is filtered before the shutdown point is reached.

The procedure is again repeated using various combinations of different prefilters and the ultimate filter. The qualities of the more open filters (serving as prefilters) can also be assessed individually.

For each filter or filter combination, the volume of liquid passed is then plotted as a function of time. Comparisons can then be made, leading to a selection of the optimum prefilter-filter couple.

If flow decay studies are carried out on the final filter (FF) and individually on each prefilter (PF), it becomes possible correctly to proportion the EFAs of the required prefilter(s)/final filter combination. This is done in keeping with the formula:

$$\text{Number of PF per FF} = \frac{\text{FF throughput per unit EFA}}{\text{PF throughput per unit EFA}}$$

Arithmetical Calculations

An elementary arithmetic example can be given illustrating the calculations involved in determining the EFA needed to filter a given batch of a preparation. An extrapolation is made from the base throughput obtained by use of a 47-mm disc. The 47-mm disc has a filter area of 1.49 inches^2. A cartridge composed of the same filter medium and porosity will be assumed to contain an area of 6 feet^2. Therefore, the volume throughput relative to a 47-mm filter of the same filter medium is:

$$\frac{6 \text{ feet}^2 \times 144 \text{ inch}^2/\text{feet}^2}{1.49 \text{ inch}^2} = 580$$

Thus, if the total throughput given by the 47-mm disc is 2500 ml, then $25 \times 580 = 1450$ I or 383 gallons will flow through the cartridge, assuming the filter medium to be similar, before it shuts down, there being 3.79 I to the gallon. The rate of flow measured on the 47-mm disc will indicate how many cartridges would be required to complete the filtration in a timely manner. However, 47-mm disc trials can only create a hypothetical, non-committal value. Any scaling with 47-mm discs has to be considered with caution due to its unreliability as regards cartridge constructions. In particular, the translation of flow rates cannot reliably be made in like fashion to pleated cartridges. Small pleated filter should be used as models for pleated cartridges.

To determine the total throughput and best filter combination one commonly utilizes 47-mm test filter composites. The first test establishes the base-throughput. It is always performed with the final filter. Multiple tests can be performed with different prefilter discs to see how the initially established base-throughput increases. Once the optimal combination is found, pleated small scale filter devices of the same combination should be used to scale the required total throughput and the size of the filter area.

As stated, the flow decay should be performed at the differential pressure and fluid temperatures to be used in the final system. This will allow the filter/fluid interaction best to simulate operating conditions. Changing these parameters, such as by the common method of accelerating the flow decay by raising the pressure, may adversely affect the accuracy of the data, due to factors such as particle impact.

By such use of disciplined flow decay studies intelligent prefilter choices can be made. These should yield filter systems with rates of flow and total throughputs that will satisfy the practical requirements of the filtrative practice.

ORDER OF PORE SIZE TRIALS

In assessing the optimizing effect of the prefilter(s) on the ultimate pore blockage of the final filter, some advocate the first use of membrane of the just-next-larger pore-size rating as prefilters to remove the small particles that are likely to cause a more sudden blockage. The more usual practice, for the same reason, is to rely upon depth-type filters rather than membranes for prefilters, but also in an ascending order of pore-size ratings.

Being of a larger pore size rating than the final filter, the prefilter will more likely permit the smaller particles to impact the final filter. The concern is that these may so resemble the pore sizes they encounter in the final filter as to cause its plugging in the fashion of a cork-in-a-bottleneck. This would lead to a catastrophic and precipitous decline in flow. To avoid this occurrence, the pore size of the prefilter is chosen to be on the smaller side among prefilters.. The tightest prefilter available is tried first to reduce the quantity of smaller particles that are passed. It should be noted, however, that the pore size rating systems are not standardized, and that depth type prefilters are, in any case, sized differently from membranes. Therefore, some uncertainty attends this effort.

The lack of information about the pore and particle sizes, and their distributions make essential the experimental assessment of the prefilter/final filter couple. It cannot otherwise be predicted. The assumption of a portion of the particle load is the prefilter's purpose. More precisely, its function is to pass only enough of the load to the final filter to provide an acceptable and timely rate of fluid flow and throughput to meet practical process requirements. In practice this means that the filter cake that builds on the final filter must remain permeable to the fluid. This necessitates avoiding both its extensive buildup and compaction. Wrasidlo and Mysels (1984) employed a 0.2-μm-nominal rated string-wound cartridge in conjunction with a 0.2/9.22-μm-rated membrane in a water filtration application. This provided the prefilter/final filter combination with only one-third the service life obtained from the same final filter protected by a 5-μm-nominal rated string wound cartridge filter.

The finding is explained as follows: Empirically it is known that filters having a comparatively large percentage of their pores in sizes similar to those of the particles tend to yield smaller throughputs. This occurrence is rationalized by an oversimplified assumption, namely, that the pore openings are round and regular, and that the particles are spherical. If that were the case, the particles that are only slightly larger than the actual pore openings would fit more precisely into them, and would plug them with great efficiency. Larger particles, like marbles being retained on a window screen, would pack differently. Although contiguous, the larger spheres would shelter those pores located on portions of the filter surface sited beneath the protective overhang of their spherical shapes. The pores within this shielded area would be less accessible to precipitous blockage by like-sized particles. To prevent the cork-in-the bottleneck situation, the succession of prefilter trials is initiated using a prefilter just larger than the pore size rating of the final filter. The object is for the prefilter to withhold a substantial portion of the particles conducive to a rapid blockage of the downstream filter.

In the subject example, the 0.2 μm nominally rated string-wound filter removed enough of the larger size particles to allow the nearly exclusive passing of the smaller ones to form a dense and impermeable filter cake. The resulting pore blockage of the final membrane thereby abbreviated its service life in terms of throughput. However, using the 0.5 μm nominally rated string-wound filter permitted a portion of larger particles to pass along with the smaller ones. In consequence, the filter cake in its mix of particle of various sizes was irregular enough in its packing pattern to remain permeable to the liquid.

If this, in fact, were to establish a general rule, then what would be required is that the prefilter should permit a proper mixture of particle sizes to deposit on the final filter. The attainment of this goal can be ascertained only by flow decay studies.

TRANSLATION OF FINDINGS

If the prefilter flow decay pattern is rather close to that of the final filter, then the brevity of the interval between the two flows offers only small possibilities for improvement. In

effect, the prefilter is itself acting as the final filter. It is too similar in its particle capture and flow propensities to offer advantages in throughput to the designated final filter. A larger pore size rated prefilter is indicated. Coupling the final filter with a prefilter serves to prolong the period of meaningful flow rates. A second prefilter, to protect the first, can give an additional period of useful life to the filtration system. In these situations, the area under each appropriate curve represents the total throughput volume. On the other hand, a very high flow rate for the prefilter being tried signals that it is too open to significantly protect the final filter; its pore size rating is too high.

HOUSING CONSIDERATIONS IN FLOW DECAY

When properly interpreted the prefilter(s)/final filter combination is extrapolated to the equipment scale needed for processing. Some general observations may be helpful. Filter manufacturers publish graphs of flow rates that are fairly linear within a range. Within this range, most of the differential pressure is being used to drive the fluid through the filter and only a small portion is used to overcome the flow resistance of the housing. When the recommended flow rate is exceeded, however, deviations can occur. First, filter efficiency and throughput may be decreased due to the high-velocity impact of particles on the filter media. Pore plugging may result.

Additionally, differential pressure will increase in a curvilinear fashion at a level higher than predicted by straight-line extrapolation. While the filter may still behave in a linear manner, the housing inlet, cartridge core, and outlet become increasingly restrictive to fluid flow, because of the relatively small orifices involved. Also, the increased frictional losses and turbulence will generate heat. This can have a destabilizing effect on proteinaceous and/or other solution components. Specifically, it has been calculated that the cartridge core or outlet pipe 3/4-inch (19-cm) can accommodate flows to a maximum of 80 l/min. Differential pressures intended to be productive of higher flow rates will avail nothing. Regardless of the higher differential pressure, the maximum rate of water flow through a 3/4-in. pipe will essentially remain 80 l (ca. 29 gal./min.).

Some filter/filter housing manufacturers list the flow resistance of their filter/housing assemblies to pure water, permitting a more accurate prediction to be made from catalog graphs relating liquid flow rates to applied differential pressures.

Filter manufacturers generally supply data concerning the rates of flow through their various filters (and housings) as a function of incremental pressure differential, for example, 4 l/min/Δ psi for a 0.2-μm-rated membrane. One must choose as a limit that differential pressure which, through the filter selected, will not exceed the flow capabilities of the filter housing. Different filter ratings (their various degrees of openness) will correspond to different ΔP levels conducive to attaining the maximum allowable liquid flow.

APPLICATION TO THE PROCESSING OPERATION

Based on restrictions of the sort just discussed, certain specific filter flow rate limitations can be recommended for the processing operation:

1. When possible, maintain a flow rate of 1–3 gal/min/ft^2 (4–12 l/min/929 cm^2) EFA.
2. The maximum flow rate should be 5 gal/min ft^2 (20 l/min/cm^2) EFA.

3. The filter cartridge-to-housing interface will normally be the most restrictive area to flow. The flow per interface should be maintained at 5–15 gal/min. (20–57 l/min) when possible.
4. The maximum flow rate per interface should be 25 gal/min (95 l/min). This implies that a single cartridge housing should have a flow rate not exceeding 25 gal/min (95 l/min) whether the cartridge length is 10 inch (25.4 cm), 20 inch (50.8 cm), or 30 inch (76.2 cm). If a flow rate of 25 gal/min (95 l/min) can be achieved with a 10 inch (25.4 cm) cartridge, increased lengths should be used to increased throughput, the service life of the filter, rather than its flow rate. Equivalent maxima for three- and seven-round housings should be 75 gal/min (285 l/min) and 175 gal/min (665 l/min), respectively.

As discussed, filter efficiency almost always is inversely proportionate to ΔP, as also most usually to the flow rate. It is directly related to service life (longevity or throughput). Thus, considerations of filter efficiency should be paramount. Subject to such dispensations as may be derived from specific validation studies, it would seem advisable to conform to the HIMA (1982) recommendations of not utilizing applied differential pressure levels of above 29 psi (2 bar).

REPETITION OF INLET PRESSURE EFFECTS

Again consider constant-pressure filtrations. When the rate of flow has decreased to one-half its original value, one-half of the filter can be said to have been consumed. When half of that flow rate is reached, another half of the remaining filter, or three-quarters of its original, has been utilized, etc. For each halving of the flow rate, there is a proportionate decrease in the remainder of yet-available filter area. The 80% diminution in the initial flow rate, at whatever constant inlet pressure level, being regarded as the economical cutoff point for a filtration, this point is reached when there has been a three- to fivefold halving of the initial rate of flow.

It makes no difference what the constant inlet pressure level is; halving the initial flow rate implies a 50% reduction in the initially available EFA. At higher inlet pressure levels, there will be larger initial liquid flows. These will more speedily supply the number of particulates that will block 50% of the filter EFA. At whatever rate the particles are supplied, however, the eventuation of the 50% EFA blockage will be signaled by a 50% decrease in flow rate. This assumes that particle deformation, or high velocity impactions caused by the differential pressure has not resulted in pore blockage.

At lower inlet pressure levels, it will merely take longer for the same volume of liquid to deliver the same number of blocking particles to 50% of the filter EFA. The flow decay curves, plots of rates of flow versus time, are dissimilar for different constant inlet pressure levels; but the total throughput volumes, the areas under the curves, are essentially the same.

Essentially but not exactly, for as already stated the effects of higher differential pressures may serve to decrease filter service life by compacting the filter cake and by tending to cause surface blockage of the filter pores by compaction of the particulates. This would attenuate the rates of flow, and, almost certainly, the throughput volume of the filter. The use of higher inlet pressure creates higher pressure differentials across the filter and produces shorter filter service lives with foreshortened throughput volumes.

There is, therefore, an economic trade-off to the use of higher inlet pressure levels. A filter's throughput volume, abbreviated to whatever extent by the effects of higher

differential pressures, is more rapidly attained at higher inlet pressures. The trade-off is between throughput volume and time. It will be advantageous to secure the larger throughput volumes characteristic of lower inlet pressures in constant-pressure filtrations only to the extent that the longer processing time is acceptable.

OVERDESIGN

There is always an area of uncertainty regarding the accuracy of extrapolations made from small trials to larger operations. Flow decay measurements are no exception. In addition to the variables of EFA and operating conditions mentioned previously, two other factors can seriously affect the accuracy of the tests. These are changes in particle distribution/burden/cleanliness of the fluid, and fluid/system dynamics. To account for these variables, filtration engineers often will 'oversize' the calculation by a factor of 1.5–2 times. Their intention is the laudable one of avoiding costly under-design, which entails interim filter changes and insufficient housing capacity. Far more economical, it is commonly reasoned, to spend unnecessarily, but not extravagantly so, for added filters than to be saddled with an installed system insufficient for its purposes. However justified the practice, it should not serve as a substitute for responsible conclusions being drawn from precise flow decay determinations. It is suggested that any appropriate correction of this type be factored into the equation after completing system sizing, rather than after the flow decay.

It is very possibly that the degree of over-design may become stipulated by the filter holder hardware. Thus, if a water purification system were to require five 10-inch (25.4 cm) cartridges in parallel to deliver the necessary flow, then a 7-cartridge housing would be indicated (assuming that 5- or 6-cartridge housings were not available in the market). Automatically, the 5-cartridge system would become oversized by two cartridges or 1.4 times. Here the degree of over-design would be permitted to rest, particularly if the next larger available housing were for 14 cartridges.

However, uncertain its numerical prescription, the practice of oversizing is prudent. It provides against the vagaries of solution variability, and seeks to avoid the interruption of critical operations.

When the system becomes sized, its EFA will usually reflect the most arduous demands, such as the peak flow rates. The accommodation of these conditions obviously requires a larger filter area than would an averaged or more modest constant rate of flow. There are engineering alternatives to such problems. Thus, the use of a storage vessel, where such a device can be tolerated and properly maintained, may permit system sizing on the basis of averaged rates of flow. The filter requirements would then be diminished.

Having said this, oversizing contains the imminent threat of yield losses due to excessive hold-up volumes. The benefits of total throughput or filtration completion assurance due to a 1.5 times oversizing, might reverse into a substantial loss of product. This balance has to be considered to have an optimal and economical solution.

SUMMARY

A flow decay is the most cost-effective method for selecting filter types, pore sizes, and filter areas, particularly in a new system. The purpose of flow decay is to provide information on the particle distribution of the fluid to be filtered, as also on the interaction

between the fluid and the filter media. Flow decays can be performed quickly and easily in "indicator trials" in a matter of hours using small disc filters, whereas foregoing their option may require experimentation with process filters for days or weeks. "Verification trials" should follow to more quantitatively confirm the results gained from using 47-mm disc filters. It is from the "verification trials" with small pleated devices that the flow modifications introduced by cartridge constructions can be evaluated and allowed for. The results forthcoming from the more focused "verification trials" are then extrapolated to the full scale pleated cartridges needed to accommodate full productions.

The completion of this series of preliminary flow decay evaluations, designed to progressively and realistically address production requirements, will provide fairly accurate data leading to the choice of proper prefilter/final filter combinations in the appropriate ratio of their filter areas.

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Filter Housings in the Biopharmaceutical Industry

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INTRODUCTION

A paradigm shift in filter housings for the biopharmaceutical industry is underway.

As the biopharmaceutical industry evolves and matures, so do the materials and designs of filter housings and peripheral equipment that serve the industry. Since the inception of the biopharmaceutical industry in the 1970s, the dominant paradigm for filtration has been to use disposable filters in reusable stainless steel filter housings.

Since the late 1990s, durable, reusable stainless steel filter housings of many sizes, shapes and capacities, are fast being replaced by a range of single-use, polymeric housings, also in a range of sizes, shapes, and capacities.

Sanitary filter housings for biopharmaceutical manufacturing have traditionally been built of 316L stainless steel, and occasionally 304L stainless steel. Biopharmaceutical manufacturing is now moving strongly to overcome some of the limitations of stainless steel filter housings through the use of a wide range of disposable polymeric housings and disposable polymeric tubing and containers, replacing traditional stainless piping and storing or mixing tanks.

The advent of disposable capsules and small disc filters for laboratory-scale applications in the 1970s sewed the first seeds of this paradigm shift. As pilot and production scale processing requirements grew, polymeric filtration assemblies grew apace. To meet biopharmaceutical batch-processing requirements, polymeric filter housings are now available in larger sizes, with configurations scalable to meet biopharmaceutical batch production needs previously met only by stainless housings and the cartridge filters used inside them.

Single-use, disposable polymeric filters, from capsules to larger encapsulated cartridge assemblies, are now displacing conventional stainless steel housings in a wide array of manufacturing applications in the biopharmaceutical industry. These filter-container systems incorporate polymeric filter housings of varying sizes in totally disposable systems (Aranha, 2004).

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We will survey key aspects of stainless steel filter housings and highlight developments prompting their increasing replacement by polymeric housings. We will highlight factors that enable disposable bioprocess container systems to overcome limitations of stainless steel housings, driving the paradigm shift (Bando 2004, Kuhn 1962).

316L STAINLESS STEEL

As in the classical pharmaceutical industry, stainless steel used for biopharmaceutical industry housings must provide high levels of corrosion resistance and be non-contaminating to process and product fluids. The material must be repeatedly cleanable and sterilizable over the service life of the housing. It must be fabricatable into industry-standard designs, durable and rugged enough to provide years of effective service life, and manufacturable at reasonable prices.

The primary metal of construction for filter housings in the biopharmaceutical industry is 316L stainless steel, classified by the Unified Numbering System as S31603. It is referred to as austenitic, that is, with a face-centered cubic crystal structure that provides a high level of smoothness and ductility; as such, it is readily molded or shaped.

Like all highly corrosion-resistant materials, 316L stainless steel is expensive. Like all steels in the early 21st century, 316L has seen marked increased in price on the world market, driven by growing world demand, most notably from China and India (American Institute for International Steel).

Corrosion Resistance

It is important to match the correct metal with its application. Metals including 316L stainless steel are subject to several different types of corrosion.

Corrosion is the result of a chemical reaction of metal with airborne oxygen to create a stable oxide, sulfide, amine, amide, sulfate, carbonate, nitrate, or other compound. As the oxide form, corrosion is the most thermodynamically stable state of the metal. However, corrosion reduces the strength and ductility of the base metal, and alters its physical dimensions.

Uniform Corrosion

Metals form a protective oxide or other compound on their exposed surfaces. The oxide layer confers the corrosion resistance of a metal, and can be only several molecules in thickness. It does not increase in thickness in stainless steel until some environmental change, such as an increase in pH or temperature, changes the equilibrium. This is termed a passive layer, and occurs in stainless steel, titanium, chromium–nickel alloys, aluminum, and zirconium.

Other metals permit the protective oxide layer to grow to a finite thickness, and then slough off the outer layer, allowing oxygen or other corrosive agents to contact the base metal. A new oxide layer forms, and the process repeats. This is referred to an active layer. Over sufficient time, the metal will be totally consumed by its environment. Active layer formation is common in cast iron, carbon steel, copper and copper alloys, and zinc.

Uniform corrosion is the uniform breakdown of the passive oxide layer, or the accelerated formation or removal of the active layer over t entire surface of the metal. Uniform corrosion is minimized by selecting corrosion-resistant materials such as 316L stainless steel (Gulliford, 1998).

Galvanic Corrosion

Metals can be ranked in order to their resistance to galvanic corrosion, according to the electrical potential measured for each in a solution of one of the metal's salts. When two metals with different galvanic potentials are suspended in an electrolyte, one metal becomes anodic, undergoes corrosion and protects the other metal, which becomes cathodic. Cathodic metals show no corrosion loss. An example is the use of zinc in galvanizing.

When the passive layer breaks down in spots, pitting results. Because there are more than two kinds or grades of stainless steel with different electromotive forces, pitting corrosion of a stainless steel is galvanic corrosion when the anode and cathode exist on the same surface. Rapid attack can occur because the cathode area is large and can create high current densities at each pit, especially in acidic chloride solutions. Stainless steels high in chromium and molybdenum have excellent resistance to pitting.

Pitting be found in pharmaceutical water-for-injection (WFI) systems is known as rouge. Rouge is an iron oxide film that forms on the surface of stainless steel in the presence of distilled or high-purity water. Rouge can originate from cast stainless steels in pump impellers and valves, vapor compression still, ground and polished surfaces, and chromium stainless steel components used in pins, discs, seals or other components requiring more strength than provided by austenitic stainless steels. Rouge can come from sources external to the filter housing, from in situ oxidation of stainless steel on mechanically polished and non-chemically passivated surfaces, and as a black oxide from surfaces in high-temperature service. Pickling or electropolishing will reduce rouge formation. Rouge can be a yellowish-green powder over a dense brown deposit on a black layer, a reddish-brown deposit over a black layer, or a dry upper crust over a gelatinous hydrated bottom layer. Rouge can be destructive to stainless steel. Rouge cannot be wiped away, and must be removed chemically, through use of electropolishing or by repassivation in nitric acid or chelant solution. The sources of rouge should always be identified and eliminated (Tuthill and Brunkow, 2004).

Another form of galvanic corrosion is crevice corrosion, caused by local differences in the oxygen concentration within a crevice. The crevice can lie under deposits on the surface, or under gaskets, lap joints, or bolt heads. Crevice corrosion can progress rapidly and is more severe with acidic halide solutions. Stainless steels high in molybdenum are often used to minimize crevice corrosion.

Intergranular Corrosion

Chromium carbides will form when austenitic stainless steel containing carbon is heated through the range of 427–899˚C (850–1650˚F.) The process is referred to as carbide precipitation or sensitization. Because it requires more chromium than is immediately available around the carbon atoms, it draws chromium from the surrounding area. This, in turn, creates an area too low in chromium to form the protective passive layer. Hence, rapid grain boundary attack can occur through galvanic corrosion. Sensitization can occur from improper heat treating, in which the entire piece is affected, or from welding, which affects a narrow band along the weld, called the heat-affected zone (Gulliford, 1998).

Stress Corrosion Cracking

For stress corrosion cracking to occur, a sensitive metal, a tensile stress and a suitable environment must all be present. Numerous alloys are known to experience stress

corrosion cracking, including stainless steel in chloride solutions. High levels of nickel increase resistance of stainless steels to stress corrosion cracking. And as the molybdenum content increases, resistance to stress cracking also increases.

Microbially Influenced Corrosion

Bacterial metabolism can generate chemical compounds that cause corrosion of stainless steel or other sensitive metals. Compounds include ammonia, carbon dioxide, hydrogen sulfide, and acids. Some bacterial metabolites can catalyze corrosion, and some bacterial concentrate manganese from the environment, which when combined with chlorine, produces hydrochloric, which can attack stainless steel (Gulliford, 1998).

316L Stainless Steel

Stainless grade 316L is most commonly used in fabrication of filter housings for the biopharmaceutical industry. While type 304 is the most commonly used stainless steel, type 316 provides the highest corrosion resistance with only a slight cost premium. It has superior corrosion resistance, due to its low carbon content, slightly higher nickel content and addition of molybdenum; it also resists chloride stress cracking. Intergranular corrosion can occur at weld points, but use of 316L stainless steel minimizes its occurrence. Pitting corrosion and granular corrosion are also less frequent occurrences with 316L (American Institute for International Steel).

Cost

Corrosion-resistant alloys are more expensive than those that corrode. The higher degree of corrosion resistance is justified by savings in downtime of fabricated equipment, processed product costs, and reduced equipment replacement costs.

The simultaneous use of 304L and 316L stainless steel is increasingly avoided by manufacturers of filter housings for the biopharmaceutical industry. The chief economic reasons are the following:

1. Traceability requirements of the industry often make it difficult for fabricators of housings to comply when both 316L and 304L stainless steels are inventoried at the housing manufacturer. When two stainless materials like 304L and 316L are inventoried in the same warehouse, it can be too easy to mix, confuse or switch these two stainless materials during the fabrication process, making strict traceability, and accurate use of stainless materials, problematic. Housing fabricators using multiple stainless steels need a robust system of traceability and accountability for stainless materials provided to the biopharmaceutical industry. This usually mandates use of highly skilled and trained workers and a robust inventory-tracking and manufacturing control system. This creates a layer of added cost that many in the highly competitive filter housing fabrication business cannot afford to incur.
2. While the material cost of 304L is lower, 316L is widely preferred in biopharmaceutical housings. Since the cost of 316L can be reduced by volume purchases, even the lower material cost of 304L is not justified, considering the potential for mix-ups and the higher corrosion-resistance and overall performance of 316L. Generally, 316L stainless steel is the dominant material for manufacture of conventional biopharmaceutical filter housings.

Recent changes in the world markets have caused stainless steel prices, including those of 316L, to leap. This has forced steel fabricators to charge substantial premiums for 316L stainless steel housings. Higher prices have in turn caused end-users in the biopharmaceutical industry to look more closely at the economics of filter housings (American Institute for International Steel).

SURFACE FINISH

Surface finishes for stainless steel are grouped under three primary classifications. Standard mechanical sheet finish is expressed in a number and letter indicating the process sequence used to manufacture the stainless steel sheet. Grit or mesh size is a second classification method, expressed as a number such as 180 grit, which specifies the smallest size abrasive used in surface finishing. A third method of classifying surface finishes is by measuring the finished surface with an instrument, typically a profilometer (Tuthill and Brunkow, 2004).

Sheet Finish

Standard mechanical steel finishes are grouped in three categories: unpolished, polished, and buffed finishes.

For filter housings, the most commonly used finish is stainless with a no. 2B mill finish. It is given a bright finish that appears smooth to the human eye, but is seen to be rough when viewed through an electron microscope.

Mill finish no. 4 is often termed a sanitary finish. It is the finish specified in the 3-A dairy standard. It is visibly grained, and when viewed with an electron microscope, is marked with numerous small ridges and valleys, each several micrometers in depth and height. The surface has no flat surfaces, and the rough, jagged edges of the ridges can break off and become a source of particulates. Not having any procedural specifications, Mill finish no. 4 can vary widely among vendors, and between lots.

Mill finish no. 7 is mirror-bright and appears perfect, to the naked eye. It is produced from no. 4 finish stainless sheet that is further finished by using cloth buffing wheels and abrasive buffing compounds, which may or may not be greaseless. Greaseless compounds contain abrasives, water and blue. Other buffing compounds contain a binder and an abrasive. The binder can be animal, vegetable, or petroleum based. When viewed in an electron microscope, Mill finish no. 7 stainless steel, most of the ridges appear to have been smoothed by the buffing process, but some are too deep to be smoothed over. These valleys or tunnels can trap buffing compounds and other contaminants that cannot be removed.

Grit Finish

Given a number that refers to the smallest abrasive grain size used in producing the finish, grit finish uses grinding and sanding abrasives that have been separated into standard particle sizes. The grit or mesh number, based on the screen apertures used to classify them into particle size categories, represents the approximate number of openings per linear inch in the final screen. These particles usually conform to standards of size set by the Coated Abrasive Manufacturers Institute.

Grit finish numbers do not have measurable quality specifications, but refer to procedural specifications. The finish can vary with the choice of abrasive materials used, hand or machine ground, and varying in duration for each grinding step. Grinding is accomplished in a series of steps, from coarser to finer. If a filter housing manufacturer

were to skip any of the multiple steps involved in achieving a fine grit finish, bearing a high grit number like 400, the housing surface might retain some of the original mill surface (Tuthill and Brunkow, 2004).

Measuring Surface Finish

Surface finishes can be directly measured by electronic instruments. Some employ a precision drive with a probe and stylus on one end. Other surface measuring devices use optical sensors and lasers (Gulliford, 1998).

Average roughness, R_a, is the most common measurement unit to stainless surface roughness measurement. It is the average value of the departures from the centerline, expressed in micro-inches. Because the R_a is an average value, surface profiles can have wide variations in "peaks and valley" yet have similar values.

Another measurement unit, R_{rms} or R_q is based on the statistical deviation of root mean square, expressed in micrometers (μm) or micro-inches (μin). This unit weights the predominant features of the stainless surface.

A comparison between finishes resulting from different grit sizes and the resulting R_{rms} or R_q values is given in Table 1.

To assign a surface finish number to a given stainless steel surface, several measurements must be taken to be representative of the entire surface, to discount local anomalies.

Chemical Surface Finishing

Pickling of stainless steel surfaces consists of immersing the metal into a bath of nitric and hydrofluoric acids, to remove surface impurities and leave the surface chemically clean and ready for further processing. Pickling produces a rough surface.

Passivation

Passivation of stainless steel is formation of a protective layer of nickel and chromium oxides that prevents formation of iron oxide, or rust. Stainless steel self-passivates, or forms an oxide layer, when exposed to air. This can be inhibited by surface contaminants such as dirt or oil, or by the presence of carbon steel particles. The passivation process is an acid cleaning process that removes these surface contaminants and permits the stainless steel to form an integral passive layer when exposed to air. Federal specification

TABLE 1 Surface Finish Comparisons

Grit size	R_{rms} (μinch)	R_{rms} (μm)	R_a (μm)
36	160	4.06	142
60	98	2.49	87
80	80	2.03	71
120	58	1.47	52
180	34	0.86	30
240	17	0.43	15
320	14	0.36	12

Source: Courtesy of Meissner Filtration Products, Inc. and Gulliford, 1998.

QQ-PP-35 outlines several passivation procedures specific to different alloys. Passivation does not improve or change the surface appearance or profile.

Electropolishing

Electropolishing uses the stainless steel to be polished as an anode in a cell holding an electrolyte. Electropolishing is the opposite of electroplating. It imparts brilliance to the surface by removing the thin oxide surface layer. It removes the surface layer of grains and embedded iron, by corroding the peaks of the surface profile, opening the "valleys" on the surface, and smoothening the surface. Electropolishing produces an extremely smooth surface, which minimizes the adherence of particulate matter on the surface.

Electropolishing removes the Bielby layer of cold-worked metal which appears to attract corrosives, including bacteria, and microstress risers. These can create poor fatigue strength, premature mechanical failure, of sometimes, stress corrosion cracking. Electropolishing removes ferrite ions from the surface, producing a surface layer richer in chromium and nickel than the base metal. The surface layer combines with oxygen to form oxides which are passive, and thicker and denser than the layer found on non-electropolished surfaces (Fig. 1) (Gulliford, 1998).

Summary

For fabricated filter housings in the biopharmaceutical industry, electropolished 316L has the best combination of properties, including corrosion resistance and cost, of available

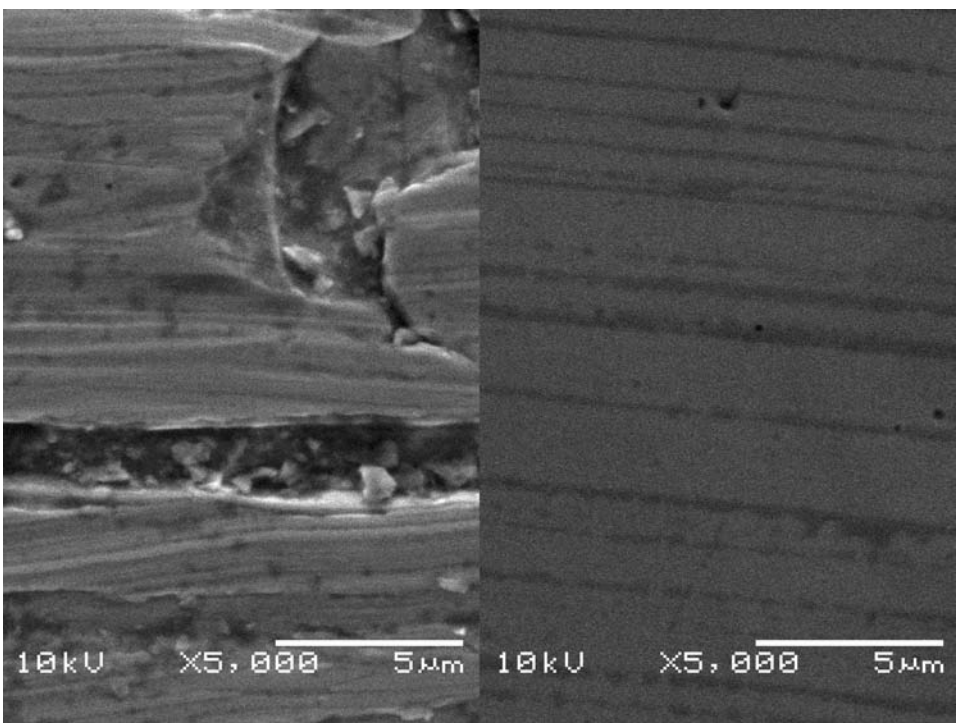


FIGURE 1 Surface finish of stainless steel (5000×) – electropolish (*left*) vs. mechanical polish (*right*). *Source*: Courtesy of Meissner Filtration Products, Inc.

stainless steel and other metals. It is by far the most widely used alloy for filter housings in the biopharmaceutical industry.

Due to the imprecision in methods used to specify surface finish, there is no single method that can suffice, even when surface finish measurement certificates are provided by the manufacturer.

Since there is a number of factors to consider when selecting the optimal material and surface finish for pharmaceutical filter housings, corrosion of the material and contamination of the filtered liquid must be considered. To minimize corrosion and contamination, the materials of construction must be compatible with the solution being filtered and the environment in which they are being used (Gulliford, 1998; Tuthill and Brunkow, 2004).

INDUSTRY STANDARDS

U.S. and international standards apply to the design and construction of stainless steel filter housings used in the biopharmaceutical industry.

U.S. Standards

cGMP

Current Good Manufacturing Practices (cGMP) mandate that all biopharmaceutical industry housings must be validated, easy to clean, be built of nonreactive materials of construction, be inert or have no effect on the product, and must be designed so as to prevent product contamination. Housing manufacturers must supply sufficient documentation to enable the biopharmaceutical manufacturer to successfully complete all stages of validation: design qualification, installation qualification, operational qualification, and performance qualification. While this may vary among housing manufacturers, such documentation can include drawings, materials specifications from metals suppliers, drawings, design calculations, equipment stages, installation operation and maintenance manuals, QC certificates, and spare parts lists. All housings must be legibly serialized and labeled, to enable full traceability (Meltzer and Jornitz, 1998).

The American Society of Mechanical Engineers

The American Society of Mechanical Engineers (ASME) develops the technical standards for the design, manufacture, and testing of pressure vessels (Tarry, 1998).

ASME's BPE Standards apply to products used in biotechnology, fermentation, and cell culture. These standards apply to equipment materials, component sterility, cleanability, finishes, tolerances and material joining.

ASME codes cite pressure limitations for various types and constructions of stainless filter housings. The codes apply to materials of construction, inspection of vessels, vessel design, stamping of the vessel, fabrication and pressure relief devices. The ASME code is widely accepted as the standard of construction, enabling biopharmaceutical companies to comply with insurance carrier standards of insurability and safety, by preventing injury claims due to faulty or substandard housings.

For non-lethal gas applications where operating pressure exceeds 15 psi and a vessel of inside diameter greater than six inches is required, housings require ASME code-stamping. ASME code-complying vessels must be built and inspected and stamped in accordance with strict ASME standards. For housings used with non-hazardous liquid filtration, when pressure exceeds 300 psi or temperature exceeds 210˚F., ASME

code-stamping of housings is required. Because many liquid filter housings will be used with integrity-testable filters, and gas for integrity testing will be applied at pressures above 15 psig, ASME code-stamping is required. In situ steam sterilization is performed on stainless steel vessels, in the range of 15–30 psig, thus bringing the vessels under the requirements for ASME code-stamping.

To manufacture filter housings bearing the ASME code stamp, a company must be approved by an insurance underwriter, which reviews the quality system, quality control, engineering design, manufacturing procedures and documentation for the facility. Approval can be given for two grades of manufacture, with stamping varying accordingly. The "U" grade stamp applies to vessels greater than 5 cubic feet in volume, with a design pressure of 250 psi, or 1.5 cubic feet in volume and 600 psig design pressure. The "U" stamp is applied by an independent ASME inspector, after review of design calculations, materials, witness of welding and of hydrostatic testing. The "UM" stamp can be placed on vessels that fall below the limits set for the "U" stamp.

P3-A Pharmaceutical Equipment Standards also apply to pharmaceutical isolation and purification equipment, chemical manufacturing equipment involved in Active Pharmaceutical Ingredients's, and equipment used for production of excipients. P3-A standards also apply to physical processing and packaging equipment (U.S. Housings Standards).

3-A Standards

3-A sanitary standards serve as the major food and dairy equipment standards. 3-A standards for the biopharmaceutical industry include that filter housings be constructed of 300-Series stainless steel or other acceptable materials, All surfaces must be capable of clean-in-place (CIP) or dismantleable for cleaning; welds must be ground smooth and flush, and free of pit cracks, crevices and inclusions; product contact surfaces must be self-draining; product surfaces must be at least no. 4 polish; no threads shall be in contact with product; non-product surfaces must have a smooth, cleanable finish; all materials must be nonporous, nonabsorbent, nontoxic, non-rusting, non-reactive, non-additive and insoluble. Type 316L stainless steel meets all these requirements and serves as the de facto 3-A standard for the biopharmaceutical industry (Meltzer and Jornitz, 1998).

European Standards

The Baseler norm was created by the Baseler Industries, Hoffmann-LaRoche, Sandoz and Ciba Geigy. While not an official standard, it specifies standards for stainless filter housings used in the pharmaceutical and biopharmaceutical industries. It specifies:

1. housings must be made from 316L stainless steel;
2. welds must be flush and polished;
3. ferrite content of the welding must be <1%;
4. the inside surface must have an average surface roughness of $R_a \leq 0.5\,\mu m$;
5. the outside surface should have an $R_a \leq 1.6\,\mu m$;
6. housing specifications must conform to common European pressure vessel regulations and 3.1B certification (www.mitc.com, www.ce-mark.com).

CE Stamp

A CE marking with the letters "CE" is affixed by manufacturers to products intended for access to the countries of the European market. "CE" is an abbreviation of the French phrase "conformité Européene." The CE marking indicates that the manufacturer has conformed to

all the obligations required by the legislation. It allows manufacturers throughout the world to freely sell industrial products to the EU markets. It affirms that the manufacturer has subjected the product to a range of tests of quality, safety, and environmental safety. Tests are implemented by directives within each EU country to regulate safety in a range of products, and are integrated into national laws in each EU country (Meltzer and Jornitz, 1998).

Many products can be CE self-certified by the manufacturer. However, EU legislation requires that an independent third party be involved in product assessment for many products, including pressure vessels such as filter housings. In case of third party certification, the approving authorities are the agencies that certify products for the European Union. These agencies, independent and for profit, are called "notified bodies." There are several hundred of them based in Europe; some have satellite operations which perform tests and submit results to Europe for final approval.

Notified bodies are authorized by European countries to serve as independent test labs and perform the steps called out by product directives, where self-certification is not possible. They must have the necessary qualifications to meet the testing requirements set forth in the directives (www.mitc.com, www.ce-mark.com).

DESIGN CRITERIA

Stainless filter housings suitable for use in the biopharmaceutical industry are generally designed to meet specific filtration application requirements. Design criteria must be specified by the end-user, and are documented to fulfill design qualification requirements. Some major design criteria for stainless housings are (Tarry, 1998):

- ultraclean interior surfaces
- high surface polish/low surface roughness
- no threaded surfaces
- low holdup volume
- no dead legs
- high level of cleanability
- complete drainability
- ease of maintenance
- optimized flow patterns to minimize shear
- secure housing sealing mechanism
- secure cartridge filter sealing mechanism
- compatibility with repeated steaming or autoclaving
- compatible with cleaning and sanitizing agents

BIOPHARMACEUTICAL INDUSTRY HOUSINGS AND APPLICATIONS

In the biopharmaceutical industry, stainless filter housings serve a wide range of applications in liquids, gases, and venting. Filter housings are installed in both "upstream" and "downstream" aseptic production areas. "Upstream" applications generally involve prefiltration or sterile filtration associated with cell and tissue culture, microbial fermentation, and "downstream" in association with such purification operations as ultrafiltration, chromatography, virus filtration, and final sterile filtration.

Housings are widely used in filtration of service fluids, such as compressed air, WFI, and for filtering a range of chemicals, including acids, bases and solvents, chemical

or solvent mixtures, and cleaning fluids. Stainless filter housings are used in batch operations, as for producing final products, and in continuous service, as in WFI purification systems.

Filter Housing Types

Disc Filter Holders

In laboratory-scale applications, where fluid volumes processed can be very small, flat-disc stainless filter holders have been used with appropriate membrane or microfibrous depth filter media, since the advent of submicron filtration. For laboratory and small-batch filtrations in the pharmaceutical and biopharmaceutical industries, flat-disc filter holders are still used to prepare small volumes of such materials as sterile tissue culture media, buffers, growth factors, sterile WFI, growth factors, active ingredients, and products such as biopharmaceuticals, vaccines, serum and blood products, and others.

Historically, disc filter holders evolved to fit standard diameters, emerging in the 1950s and 1960s, when the predominant early hydrophilic filter media were mixed esters of cellulose, a highly fragile and brittle material that cannot be corrugated into pleated configurations, as can later polymers used for microfiltration. As a consequence, the only way to increase flow, throughput volume and processing speed with these brittle early discs was to increase the diameter of the discs and disc-holders, and to use multiple-disc or multi-plate housings.

The industry standard diameters for dual-plate, pressure-driven stainless filter holders, usually mounted in stainless steel legs, that emerged in the 1960s and 1970s were: 13, 25, 47, 90, 142, and 293 mm. While dual-plate, sanitary stainless filter holders were commonly used for small-batch filtrations, disc filters were also deployed in 47-mm glass filter holders utilizing vacuum for water microbiology and other analytical applications, in which the microbial or particulate retentate on the filter surface is counted or otherwise analyzed, in 47-mm stainless steel holders for vacuum filtration or analytical applications, and in inline, polypropylene holders for pressure-driven gas or liquid applications. Disc filter holders were also constructed of polycarbonate, polytetrafluor-ethylene (PTFE) or polypropylene, for non-sterile applications.

For sterilizing applications, the stainless filter holders were and are preferred for their autoclavability. To prevent membrane sticking to heated interior surfaces, PTFE is commonly deposited on the inner aspects of the upper (upstream) and lower (downstream) disc filter plates in sanitary disc filter holders.

In the stainless steel filter holder, usually manufactured in diameters of 90, 142, and 293 mm, 316L stainless is the standard material of construction. The design incorporates a stainless base plate with sanitary outlet connections, mounted on three anodized aluminum legs, with rubber tips for adhesion to benchtop surfaces on the 90 and 142 mm sizes, and nylon pads on the 293 mm size.. A porous screen, usually photo-etched and coated with PFTE for sterile applications, lies atop the base plate, serving to support and drain the filtrate. An underdrain support is installed beneath the support screen.

The disc filter is placed atop the support screen, and a sealing O-ring acting to prevent bypass around the filter disc. Over-tightening of the sealing O-ring can cause deformation of the O-ring with permanent damage, leakage of the system under pressure, and possibly deformation and damage to the membrane filter disc. The two plates are bolted together with sanitary bolts and handwheel knobs or T-bolts, tightened in alternative sequence, for sterilizing applications, or Allen head cap screws for non-aseptic applications. Inlet and outlet connections are TC sanitary for sterilizing applications, or stepped hose barb for non-aseptic applications. A spring-loaded, sanitary vent valve is

built into the top plate, to release air that becomes trapped, upstream upon introduction of liquid; this prevents partial or complete airlocking of the filter chamber, which would slow or completely stop the flow of liquid through the wetted filter (Fig. 2).

In sterilizing applications, the membrane disc is wetted and installed inside the stainless holder, then integrity tested one the unit is sealed. Upon passing the bubble point or diffusion test, liquid is introduced through the top, inlet plate. The vent valve is kept open until unfiltered liquid is seen to exit through it, then it is closed for filtration. The upstream vent valve can also be used for sampling of unfiltered liquid. Some plate disc holder models are designed with downstream drain/sample valves, to enable sampling of the filtrate during filtration, or to ease drainage and cleaning of the filter unit after use (Table 2).

Plate-and-Frame Filter Presses

Depth filtration media is widely used in the biopharmaceutical industry for cell harvesting and other applications where high solids-loading occurs. Traditionally, plate-and-frame type filter housings have been used for these applications, compressing sheets of flat-sheet depth media into a flow configuration that enables high volumes of solids to be removed from the liquid stream.



FIGURE 2 Stainless steel disc filter holder, showing upstream and downstream support screens, sanitary connections. This reusable unit can be autoclaved. *Source:* Courtesy of Meissner Filtration Products, Inc.

TABLE 2 Disc Filter Holders: Filter Diameters and Typical EFA's

Disc filter diameter, nominal (mm)	Effective filtration area (approx.), typical (cm^2)
4	0.1
13	0.8
25	3.9
47	13.8
90	45.5
142	127
293	518

Source: Courtesy of Meissner Filtration Products, Inc.

While these filter presses or plate-and-frame filters are not usually referred to as housings, because they are not enclosed in a stainless steel shell or housing bell, they are utilized in the biopharmaceutical industry and both the filter presses and flat-sheet media are manufactured for and are used widely in the industry.

Major advantages of such systems include availability of a wide variety of filter sheets, of varying construction and efficiency, tailored closely to the needs of the application. Cost-per-unit of filter surface is lower than that of cartridge-type depth filters or lenticular depth filters of similar removal efficiency ratings. Plate-and-frame systems are also readily expandable and scalable up or down, in case of process expansion or downsizing, by adjusting the number of discs and therefore the surface area of the depth filtration media.

Major disadvantages include their open-air format which can permit ingress of airborne contaminants, adsorption and leakage of valuable product or process fluids, which must be collected in a drip pan underneath the filter pads and returned to the process stream or discarded, and the cost of labor to install, prepare, operate and clean the filter press, and dispose of spent filter sheets.

Stacked Disc Housings

Stacked disc filter housings are manufactured by a range of manufacturers. They are commonly in applications used where depth filtration media is employed to remove relatively high levels of contaminant suspended solids, such as cell harvesting after cell culture. A range of depth media, some with surface-charge-enhanced removal properties, is employed inside (www.millipore.com).

Depth media in stacked disc housings is configured in circular discs, supported and encapsulated inside lens-shaped, or lenticular, polymeric support structures. These depth filtration media discs, called stacked cartridges by some manufacturers, have a flow pattern that permits liquid inlet through the outsides of the lenticular support structures, and filtrate outlet through a central support and drainage post. The ten-inch diameter lenticular discs are stacked up on the center post, often five or six high, to provide sufficient flow rate and throughput for processing batches volumes. Lenticular disc filter stacks can be as high as forty inches. The stack, sized by preliminary filterability and scale-up testing, is firmly held in place by a spring-loaded compression tool. With several leading manufacturers of this technology, the discs are generally designed to fit only the same manufacturer's filter housings. Adapters are available if discs from one manufacturer are to be used in another manufacturer's housing (Pharmaceutical Housings, Millipore).

Configurations of stacked disc housings typically include T-style sanitary TC inlet and outlet connections attached to the bottom of the base plate. Housing upper portions,

often called domes or shells, are usually fitted with handles, and combined or separate sanitary vent and gauge ports atop the shell, and drain ports usually located on the (outlet) elbow attached to the base plate. Wetted surfaces are generally electropolished to an R_a of 20 μinch finer to minimize adhesion of bacteria or trapping of particles. The entire housing assembly is mounted atop three or four stainless steel legs, depending on the housing size and manufacturer's design, each leg being adjustable in length.

Pod Assemblies

A novel, scalable filter system that bears some resemblance to both plate-and-frame filter press systems, and to lenticular stacked discs, the "Pod" system design includes a steel frame, in two expandable holder sizes, and pod-shaped filtration modules in three sizes. The frame surrounds and restrains pod-shaped filter elements which are arrayed in racks scalable from 0.1 to 5 m^2 for pilot operations, and from 5 to 30 m^2 for process scale operations. The process scale holder holds from 5 to 10 pods per rack. Up to three racks can be stacked to provide from 5 to 30 m^2 of filtration area, depending on processing requirements. Filter media is multi-media graded density depth filtration media, with adsorptive media available, or additional layers of microporous cellulosic media, for clarification, solids removal and protection of downstream filters and equipment (Millipore Pod System).

The pilot-scale system uses 304L stainless steel, for metal surfaces not in contact with process fluids. The process scale pod system uses 316L stainless steel for all liquid-contact surfaces, electropolished stainless to R_a <0.4 μm (<15 μinch) and 304L stainless steel for metal surfaces not in contact with process fluids. Silicone gaskets are used for sealing the pod assemblies.

Advantages of a pod system include scalability to meet specific process flow and throughput requirements, and compressing of filtration modules in a compact, modular design. This permits easy change-outs and disposal of the pods, eliminating the need for hardware cleaning and cleaning validation, and shortens turn-around time. Disposable adapters permit connection of modular pods to process piping. The self-contained system protects operators from exposure to biohazards.

With its stainless frame and disposable pod filter elements, the pod system design stands as something of a design hybrid, a bridge between traditional filter presses with their stainless steel frames, lenticular cartridges with their stacked disc housings, cartridge filter housings, and the new disposable processing systems. Pods are a scalable, modular, flexible system for clarification and prefiltration applications using depth, adsorptive and microporous filter media in a unique, podular design.

Cartridge Housings

The mainstay of traditional filter housings in the biopharmaceutical and parent pharmaceutical industries has been the 316L stainless steel cartridge filter housing (Meltzer and Jornitz, 1998).

Cartridge housings for biopharmaceutical filtration are generally sanitary in design, having no threaded surfaces or dead-legs in which contaminants can collect. Cartridge housings are designed according to their liquid flow-path. In a T-style design, liquid enters from the bottom, flows up and swirls around the cartridges, and the filtrate flows into the center of the cartridges and out of the housing through the port in the center of the housing. In inline style designs, liquid enters the housing from one end, flows through the filter cartridge or cartridges, and the filtrate exits the housing at the opposite end (Messner Housings Design; Millipore Housings Design; Pall Housings Design; Sartorius Housings Design).

Both T-style and inline designs have inherent advantages and disadvantages. T-style housings can be installed, CIP, steamed in place (SIP), and the cartridges changed out, while leaving the housing in place, without disconnecting it from its piping. The fixed base and inlet and outlet connections facilitate skid-mounting, as do the fairly short piping dimensions, and the ability to scale-up to larger cartridge filters by utilizing a longer housing shell, also referred to as the bell or bowl. The shell requires a vent valve, usually a sanitary ball valve, to remove air, a pressure gauge port for monitoring inlet pressure on the upstream side of the filters, and usually sharing the pressure gauge port, a connection for an automatic filter integrity test instrument. A drain port will be installed either on or adjacent to the inlet elbow on the base plate, or occasionally on the side of the shell (Fig. 3).

Because their inlet and outlet connections are located at either end of an inline-style housing, these housings must be disconnected and removed from their surrounding piping, taken off-line, and their cartridge filters changed out and new cartridges reinstalled, before reconnecting and steaming in place. Inline housings have the advantage of direct, straight-through flow of liquids or gases, and thereby cause a lower pressure drop across the housing-cartridge installation. In T-style housings, liquids or gases are forced to flow around right-angles at both the entry and exit connections (sanitary flanges), thus increasing overall pressure drop across the housing-and-cartridge installation (Fig. 4).

To accommodate higher flow rates and produce lower differential pressures and longer service life, multi-round cartridge filter housings are designed to accommodate multiple cartridge filters. Accordingly, the housing base, shell, internal cartridge connections, and external clamp sealing mechanism are enlarged proportionately.

Housing Adaptations to Applications

Filter housings are employed in a wide variety of bioprocess streams. Some of these applications require adaptations to meet special requirements (Tarry, 1998).

Sterile vent filter housings. For sterile vent filter installations, heat-jacketed or electrically heat-traced designs are commonly used. Jacketed designs incorporate an extra, outer stainless steel "jacket" that provides a leakproof layer of insulating or heating fluid, such as glycol for maintaining constant liquid temperatures, or steam for maintaining elevated temperatures. In venting applications, it is critical to minimize steam condensate to prevent blockage of the filter pores and creation of a vacuum inside the housing. Condensate can be drained to a steam trap, or through drains located in the base plate or the drain of a housing. Because tank vent filters are commonly changed-out every several months, or even annually, inline-style housings can be used, minimizing the inconvenience of change-outs. It is important to match the pressure rating of the steam jacket to that of the unregulated plant steam supply; alternatively, a pressure relief valve rated at a pressure below that of the steam jacket can be installed. Steam jacket pressure should be kept as low as possible, to minimize damage to cartridge filters inside the housing. For both jacketed and heat-traced housings, heat should be turned off when the filters are not in use, to minimize oxidative degradation from dry heat (Fig. 5).

Heat tracing utilizes electrically based, grounded heating elements, for applications where a constant, elevated temperature is required for liquid inside the filter housing. Electrical heat-tracing is attached to the housing shell, with either self-regulating heat-tape or a process controller with a thermocouple.



FIGURE 3 T-style sanitary housing. *Source*: Courtesy of Meissner Filtration Products, Inc.

Virus filter housings can be multi-chambered, incorporating separate chambers within a larger housing, to permit segregation of individual virus-retentive filters for the purpose of integrity testing.

Stacked-disc housings. Small-volume sterile or particulate filtration of liquids or gases can be performed with small stacked disc filter housings. These permit use of stacked disc filters inside sanitary 316L stainless steel housings, for removal of microbial and particulate contamination from bioprocess fluids (Stacked Disc Fitter Housings, Millipore).

Small-flow filter housings. Small-flow or small-area "mini-cartridge" filter housings permit use of small-surface-area cartridge filters in laboratory and pilot scale operations. Designed in both inline and T-style designs, they are used in prefiltration, particulate removal and sterile filtration of liquids and gases.

Fermentor air and gas sterilization housings. For sterilizing gases and maintaining the sterility of fermentor inlet air, stainless steel housings are designed to provide up to 10 bar (147 psig) pressure. Some of these housings are designed in a wind tunnel, to streamline them in order to reduce pressure drop across the installation, lowering



FIGURE 4 Inline-style sanitary housing. *Source*: Courtesy of Meissner Filtration Products, Inc.

operating costs. These housings are steam-sterilizable and most frequently use the Code 7, bayonet-style connection for maximum reliability in applications which can range in duration from days to months or longer.

Multi-Round Housings

To provide greater flow, longer service life and generally increased filtration capacity, filter housings with multiple filters flowing in parallel are designed to hold 3, 5, 7, 9, 11, 12 and 21 filters. Sanitary designs utilize a receiver plate into which the cartridge filters are secured in sanitary cups, usually double-O-rings with bayonet locking tabs and size -226 or double-O-rings with -222 size. A center tie-rod is commonly welded into the receiver plate and extending to the top of the cartridges, often connecting to a spring-loaded compression plate. This plate is designed to be thread-free and surrounds the top of the cartridge, which often has a locator fin that can be held in place loosely by the compression plate. Tolerances in the openings of the restraint plate allow sufficient room to permit the cartridges to expand or "grow" in length when they are SIP or sanitized with hot water (SIP). By contrast, many single-round, one-cartridge filter housings incorporate a shell or bowl design that fits fairly snugly against the top of the cartridge filter, restraining it so that it does not "pop out" of its receptacle in the receiver plate, while allowing sufficient space for the cartridge to "grow" when heated (Messner Housings Design; Millipore Housings Design; Pall Housings Design; Sartorius Housings Design).



FIGURE 5 Steam jacketed housing for sterile vent installations. *Source*: Courtesy of Meissner Filtration Products, Inc.

In multi-round sanitary housings, double O-ring seals are almost universally employed in sterile applications, to provide a redundant seal against bypass around the cartridge filters. Gasket seals may generally be employed in less-critical applications, including non-aseptic clarification and prefiltration of liquids.

HOUSING SEALING MECHANISMS

A variety of clamps is used to seal sanitary housings. The three-part sanitary clamp is widely prevalent, with a ferrule and gasket assembly. V-band clamps are commonly used in non-aseptic applications, such as chemical or solvent filtration.

Single-round housings most often use sanitary clamps, to facilitate opening and sealing of the housing, and silicone O-rings are used forming the seal between the base plate and the shell, when aqueous liquids are being processed.

Multi-round housings often use V-band or swing-bolt closures, or occasionally, cam lock closures.

As elastomeric O-rings will deform upon repeated use, they must be checked regularly and replaced before shape distortion or other damage causes leakage or false integrity test failures. O-ring materials in common use are silicone, EDPM, Viton, and PTFE.

Cartridge-Sealing Mechanisms

Three predominant sealing mechanisms are used for 10-inch, 10-inch-multiple, and many smaller cartridges. All cartridges, large or small, considered sanitary and suitable for aseptic applications are single-open-ended in construction, most frequently with a locator or "bomb" fin atop the closed end and double O-rings forming the seal at the open end.

The 226 size double-O-ring with opposing locking tabs (Code 7, double bayonet) is the state-of-the-art for sealing cartridge filters. Its twist-lock feature protects against dislodging of the cartridge, while its double O-rings give redundant protection against filtration bypass.

The 222 size double O-ring design, (Code 8, plug-in style) is slightly smaller in diameter than the 226 bayonet-locking style. It is still widely used in aseptic and non-aseptic filtrations. Multi-round housings for Code 8 style cartridge closures are frequently equipped with spring mechanisms for multi-round housings or the shell length is sufficiently snug to restrain the cartridges in place during operation, cleaning or steaming. These dimensions vary somewhat by manufacturer, and retrofit cartridges should be tested for snug fit in housings from other manufacturers.

Knife-edge or flat gasket-seal cartridges (Code F) can be either single- or double-open-ended. This cartridge sealing mechanism is more subject to installation error and is less secure and reliable than Code 7 or Code 8 connections, knife-edge/flat gasket seals (Fig. 6).

Housing Connection Styles

Sanitary flange, threaded (non-sanitary), RF raised-face flange (non-sanitary), and butt-weld (non-sanitary) (Fig. 7).

VENT AND DRAIN VALVES

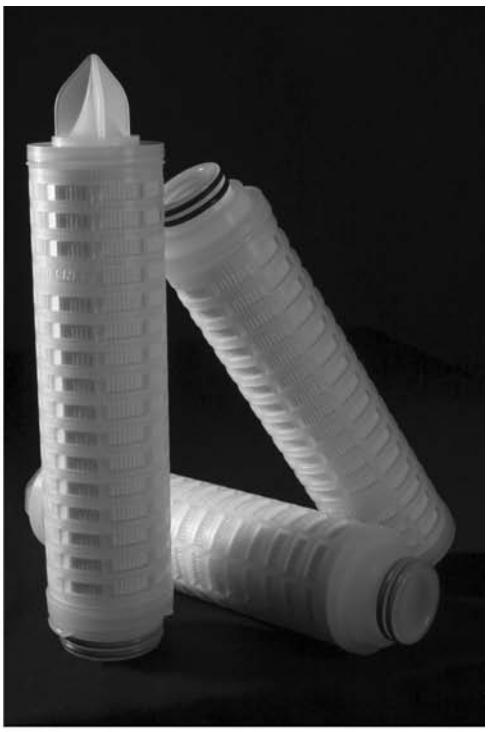
Vent valve designs include sanitary spring-loaded, diaphragm valve and plunger type.

Typical vent valve designs are shown in the attached photograph. The threads of sanitary vent valves are isolated from contact with filtered liquid or gas by an O-ring on the valve stem. The valve has a center channel by which fluid exits the housing. At the tip of the valve is a PTFE tip, which is backed-off to permit venting or draining.

TYPICAL OPERATING SPECIFICATIONS

Housings are designed to operate over a wide range of pressures and temperatures. Housing manufacturers provide ratings, either ASME- or non-Code, for both liquid and gas operation.

Typical liquid pressure ratings (maximum operating limits) of liquid filter housings is 150 psig, or 10 bar.



(A)



(B)

FIGURE 6 (**A**) 222- and (**B**) 226-size double O-ring seals shown with 10-inch cartridges. *Source*: Courtesy of Meissner Filtration Products, Inc.

Housings may be operated under partial vacuum conditions, and the manufacturer must supply a rating stating the suitability of a given filter housing to operate under partial vacuum conditions.



FIGURE 7 Housing connection styles include sanitary, non-threaded; threaded (NPT); and raised-face flange. *Source*: Courtesy of Meissner Filtration Products, Inc.

Temperature ratings for stainless housings are generally not specified. It is assumed that all stainless vessels are capable of withstanding the temperatures required to sterilize filters, usually 121–135˚C.

INSTALLATION AND MAINTENANCE CONSIDERATIONS

Manufacturers of stainless steel housings generally provide extensive installation, operation and maintenance manuals for use with the housings. Several key considerations emerge.

Assembly and installation. All component parts must be correctly oriented and installed in fixed or flexible plumbing. After assembly, the housing must be disassembled so that all fluid contact surfaces are clean prior to use. Base plate O-rings must be properly installed and clean prior to use. Cartridge filters must be carefully and correctly inserted to ensure correct functioning and to prevent damage. The shell must be clean and correctly installed atop the housing base plate, with the correct clamp installed and sufficiently tightened to effect a reliable seal, but not over-tightened so as to compromise the seal.

Startup operation. After partially opening the inlet and outlet valves, the housing vent valve must be opened until liquid fills the housing, then closed. Inlet and outlet valves are now fully opened to ensure full flow.

Cartridge replacement. Stainless housings must be depressurized and drained before changing out cartridge filters. If cartridges are to be reused, they must be handled with care to prevent damage to the filter, connections and O-rings that could compromise performance on subsequent usage. Base plate O-rings must be removed, inspected for wear and damage, and cleaned. All fluid contact surfaces must be cleaned before steaming and reuse. New cartridges require careful handling, to prevent damage to connections, O-rings and filter.

Valve maintenance. Fluid contact surfaces, including valve stem and O-ring, must be cleaned before reuse. Damaged or worn O-rings must be replaced and cleaned. The PTFE valve seal tip must also be cleaned and monitored for damage, wear and eventual replacement.

Cleaning. All surfaces must be cleaned with hot purified water an a suitable non-abrasive detergent. Where water and detergents are inappropriate, a compatible solvent or cleaning solution can be used. Where housing O-rings will be reused, they must be washed with hot water and detergent. All components must be thoroughly rinsed with purified, filtered water, and then allowed to air-dry in a clean environment, or blown-dry with clean, filtered air.

Operating recommendations. To ensure safe, reliable operation of sanitary stainless filter housings, several important steps must be taken:

- Filter cartridges should be replaced before flow is significantly reduced or limiting differential pressure is reached.
- Housings should never be opened without being sure that pressure is turned off, the housing depressurized and process fluid drained.
- Cartridge filters used in aggressive, corrosive, toxic or otherwise potentially dangerous liquids should always be examined for damage, prior to reinstallation and use.
- The housing should be routinely cleaned to remove particulate and any accumulated debris
- The housing must always be cleaned before filtering a different fluid.
- Avoid damaging or scratching all sealing surfaces.
- Inspect all parts, particularly O-rings, clamps and T-bolt assemblies for possible damage and/or excessive wear.
- All worn or damaged parts must be replaced promptly.
- Keep spare O-rings and a valve seal kit available at all times.
- Be sure the housing clamp is properly positioned and fully seated and tightened before pressurizing the housing.
- Periodically vent the housing to released entrapped gas, when filtering gaseous process liquids.
- Open and close all system valves slowly.

Steam sterilization (SIP). Housing manufacturers supply detailed instructions for safe and effective steam sterilization of sanitary stainless filter housings. These instructions must be followed carefully to ensure safe and effective sterilization, and to prevent damage to cartridge filters and operators.

Autoclave sterilization. Manufacturers provide detailed instructions for effective autoclave sterilization of smaller sanitary stainless filter housings. As with steam sterilization, recommended procedures must be carefully followed to ensure effective sterilization and prevent damage to filters.

Use of stainless steel with ultra-smooth product-contact surfaces with stringent CIP and SIP procedures have minimized if not eliminated the filter housing as a source of process and product contamination.

Integrity testing. It is recommended that all users of sanitary stainless steel filter housings for sterile applications use automated integrity test instruments for integrity testing of cartridge or disc filters. Procedures for operation of test instruments are provided by the major filter manufacturers and must be carefully followed to ensure effective, reliable operation.

Validation. For sterile filtration applications, steam and autoclave sterilization cycles must be validated to ensure reliable sterilization and to comply with cGMP requirements. Cleaning procedures must also be validated for aseptic applications.

Cost factors. Some significant costs associated with operating sanitary stainless steel filter housings include:

- labor for installation, operation, cleaning, and maintaining housings
- labor for CIP and SIP operation,
- capital costs of CIP equipment,
- capital costs of SIP equipment,
- energy for CIP and SIP operation,
- cleaning chemicals,
- disposal of cleaning chemicals, and
- labor for validation of cleaning procedures including analytical procedures, testing, reporting, presentation to regulatory authorities.

MARKET TRENDS AND TECHNOLOGY CHANGES

The inherent limitations of stainless steel filter housings created a need—and therefore an opportunity—for alternative configurations and materials to satisfy those needs, and to irreversibly alter the way in which many fluids are filtered in the biopharmaceutical industry.

The decision to install stainless filter housings is increasingly weighed in light of necessary operating costs, which include: labor for setup, operation, dismantling and cleaning; cleaning and sanitizing chemical consumption; labor and analytical resource consumption in cleaning validation; energy consumption in steam sterilization; operator safety concerns in handling toxic or dangerous fluids processed by disposable filters in reusable filter housings. This evaluation is also made with growing industry adoption of the growing number of standard and custom configurations and sizes of disposable filters that can perform the same filtration processes as stainless steel filter housings, usually with greatly reduced operating costs.

Some of the key factors underlying the paradigm shift in housings in the biopharmaceutical industry—from the point of view of stainless steel filter housings—are surveyed here. Numerous authors have documented the new technologies that have enabled the increasing use of disposables in the biopharmaceutical industry. The economics of the shift have also been well documented by numerous authors (De Palma 2004; Rios 2003; Sinclair 2002).

The paradigm shift in housings in the biopharmaceutical industry is rooted in the numerous limitations of stainless steel housings technology. They have served well and long, but the dynamic biopharmaceutical industry has numerous requirements that stainless housings cannot provide.

While stainless steel housings have served the biopharmaceutical industry since its inception, the need of the industry for a flexible, versatile, lower-operating-cost, expandable, up-scalable, down-scalable housing-and-filter configuration have loomed

ever larger. As the biopharmaceutical industry matures, it needs a more responsive, adaptable and cost-effective technology than the rigid housings-and-related-plumbing of the stainless steel housing. The industry needs more versatile, quickly and easily installed, pre-fabricated, pre-sterilized or autoclavable polymeric filter housings and related apparatus. In this technology arena, the industry needs faster instead slower, easier instead of more difficult. Increasingly, it needs disposable more than it needs reusable.

Stainless steel economics. In the first decade of the twenty-first century, stainless steel prices jumped as much as 30% annually in world markets. The rising economies of China and India are frequently cited as major contributors to rising demand for stainless steel markets, with resulting large increases in steel in world market prices. Some of the direct results have been that, first, the prices of stainless steel housings have virtually doubled from 2001 to 2006, and second, that filter housing manufacture is increasingly taking place in India and other countries where labor costs are much lower than in the United States and Europe. The price of steel thus encourages both attention to the costs of stainless filter housings and a shift in the locus of stainless housings manufacture to emerging market nations (American Institute for International Steel).

Delay of installation. Design and construction of hard-plumbed facilities in general, and stainless steel housings and related filtration plumbing and hardware, are time-consuming processes. Design, approval, construction and installation of equipment in hard-plumbed biopharmaceutical facilities can require a span of one or more years. When time spent in facilities validation is added, the time requirement is substantial. With economic pressure on smaller biopharmaceutical companies to reduce time-to-market, stainless steel housings are again part of the problem, not the solution.

Logistical Inflexibility. Stainless steel filter housings and related piping, valves, connections, etc. are inherently rigid and fixed installations. The hard-plumbed housing is part of a highly inflexible manufacturing operation that also includes stainless tanks, CIP and SIP equipment, "permanent" installations with nearly complete inflexibility. In the face of the biopharmaceutical industry's growing need for rapid plant expansions, mobility, portability, and rapid change-overs of processing equipment, rigid, hard-plumbed installations, including stainless steel filter housings and peripheral piping, valves, tanks, etc. do not serve the industry optimally. Where the industry needs fast scale-up and scale-down of manufacturing operations at both biopharmaceutical manufacturers and at contract manufacturing organizations (CMOs), the inflexibility of the stainless steel filter housing is once more part of the problem, not the solution.

Validation. With increasing FDA attention to cleaning validation in the early twenty-first century, biopharmaceutical manufacturers are forced to examine the costs in labor and time spent on validating the cleaning of stainless steel housings, along with the stainless or other rigid plumbing and hardware associated with them (Rios 2003; Parenteral Drug Association, 1998).

Cleaning validation involves many hours of time spent by high-salaried technical staff, in providing a scientific basis upon which to justify use of cleaning techniques and chemicals in assuring that reusable equipment, including stainless steel housings, does not cross-contaminate subsequent batches of product, and does not contaminate it with cleaning chemical substances. The scientific rationale and testing, as routine cleaning records, must be thoroughly documented and ready to discuss and defend to FDA or other regulatory authorities. FDA's requirement does

not specify cleaning procedures, but mandates that the biopharmaceutical manufacturer provide a scientific basis for the procedures (FDA, 2004).

Where filtration is an integral part of a process requiring cleaning, stainless steel filter housings and related plumbing and their cleaning are a significant component in the work of validating manufacturing cleaning procedures and practices.

CIP and SIP cycles for stainless steel filter housings and cartridge systems must also be validated. These, too, are time-consuming validation processes.

Installation and operating considerations. As outlined above, numerous time-consuming steps are required for initial installation and ongoing operation of stainless steel housings. Operators must learn and carefully execute installation, filter changeouts, valve and O-ring inspection and changeouts, inspection and repair of damaged or worn parts, cleaning procedures, CIP procedures, SIP procedures, integrity test procedures, and important aspects of stainless filter housings – operating conditions, cleaning sterilization (by inline steam or autoclave, depending on size of the housing and installation plumbing) procedures – must be validated. The installed housing and attendant plumbing and hardware are generally fixed, if installed in place and immobile, unless mounted on a skid, although such units are not frequently moved once pressed into service. Stainless steel housings with T-style connections can be scaled-up by using a longer housing shell, if the surrounding installation hardware and plumbing allows space to do so.

Scale-up limitations. As biopharmaceutical products move through the stages of product and process development during Phases I, II and III Clinical Trials, a filtration system centering on stainless steel filter housings and related fixed equipment such as pumps, valves, piping, and connections can be slow and expensive to scale-up.

Summary of issues with Stainless Filter Housings

A long list shows of technical and economic considerations inherent in the structure, function and economics of stainless steel filter housings drives the paradigm shift from stainless housings to disposable systems.

Chief among these issues are:

- Rapid and substantial price increases in stainless steel
- Time delay and cost of initial design and installation
  - Engineering designs and approvals
  - Facilities approvals and regulatory approvals
- Cost of cleaning validation
  - CIP cycles
  - Sterilization
- SIP cycles
  - Integrity testing
- Labor-intensity of operation
- Cost of maintenance
- Cost of cleaning
- Logistical inflexibility of fixed installations

- Difficult to scale-up
- Difficult to scale-down

THE NEW HOUSING PARADIGM: DISPOSABLE BIOPROCESS CONTAINER SYSTEMS

Plastic filter holders are not new to the biopharmaceutical industry, or to other industries. Since the first syringe and capsule filters appeared in the 1970s and 1980s, they have achieved wide usage, primarily in small-scale filtrations at the laboratory and pilot scale, for both liquids and gases (Small disposable capsule filters, Millipore).

Unlike cartridges, disposable capsules do not require emplacement in external steel housings. They are available in many sizes and types of filter constructions, whether disk, multi-disc, pleated cylinders of various lengths and of different effective filtration areas. They are smaller in filter area than either standard or mini-cartridges. The EFA of these devices is on the order of 0.01–1.8 m^2. Disposable filter syringes range from 4 through 25 mm diameter discs, attached to syringes as both a liquid reservoir and pressure source, to small, pleated capsule filters commonly ranging from 0.3 to 2 ft^2 in EFA. As described below, encapsulated cartridges offer the EFA of up to four 10-inch cartridges in a high-capacity capsule assembly.

When intended for air and vent filters, capsule filters incorporate hydrophobic polymeric filter materials, including polyvinylidene fluoride, polyethersulfone, polypropylene and PTFE. The membrane and its support and drainage layers are encapsulated within a shell molded of some suitable polymer, such as polycarbonate, polyethylene, or, most often, polypropylene. The use of transparent polymer, for example, polycarbonate, allows the filtration operation to be visibly monitored. The capsules' materials of construction are identical to those of the cartridge and mini-cartridge, and are integral with ready-to-connect sanitary fittings, drain plugs, and vent plugs. Their designs and constructions functionally match those of stainless housings of comparable capacities (Fig. 8).

Disposable capsules are autoclavable, but are generally not in-line steam sterilizable. They are available presterilized by gamma radiation, steam or by ethylene oxide, and may be stored ready for use. Certification of effective sterilization by the filter manufacturer should be in the user's possession, available for regulatory inspections. In, addition, validation details concerning extractables, and bacterial retention studies should also be on hand at the user's location.

The use of disposables entails very little setup time, and virtually no clean up time. Cleaning validation, which must to be performed with fixed equipment like filter housings, is greatly reduced. Disposable filters do not require such cleaning regimes, and therefore the validation of cleaning exercises is avoided.

This is especially so in small scale operations; for example in conjunction with disposable bag systems. A disposable capsule filter is connected to a disposable bag. Both are available in different sizes for specific application requirements. Once the capsule filter is connected, the bag and filter are gamma irradiated to sterilize the entire set-up. Filtered product remaining on the surfaces of the filter cartridge and housing may need to be avoided due to health hazards or biological activity. Disposable systems can be replaced without coming in contact with the product.

With capsules or cartridges of any size or construction, the identifying description recorded on their shells extends as well to the incorporated filter material. This aids the



FIGURE 8 Capsule filters. *Source*: Courtesy of Meissner Filtration Products, Inc.

record-keeping that the FDA requires. In the case of conventional 293-mm flat discs, once removed from their shipping containers, their identification can be misplaced or lost.

The cost of the disposable units is reduced by the savings in setup and cleaning time and labor. The increase by filter manufacturers in the tailoring of disposable filter devices to specific usages further explains the popularity of their acceptance, and foreshadows their idea usage.

The news—and the basis for the paradigm shift—since the late 1990s is the great expansion of configurations and scale of disposables. New and larger sizes and shapes have been designed and implemented to offer a broader range of choices available to the biopharmaceutical industry.

Larger-Scale Capsule Assemblies

In the late 1990s and early twenty-first century, major filter manufacturers introduced high-capacity filter capsules, which are essentially encapsulated cartridge filters in a self-contained shell, ready for use. These high-capacity capsules solved at least two needs, first, the need for a disposable (capsule) filter that had higher capacity (flow, service life) than standard capsule filters, and second, the need for small production-scale filtration

that is free of many of the encumbrances and limitations of stainless steel filter housings (Fig. 9) (Bardo, 2004).

These high-capacity capsule filters are at once capsules and cartridges, filters and housings. As in integral, preassembled unit that can be connected to tubing, hose or piping, and available in either T-style or inline configurations, they are equivalent in most respects—but not all—to cartridge filter-and-stainless steel housing systems of the same lengths, usually 10, 20, 30, or 40 inches.

While encapsulated cartridge assemblies are not yet available in multi-round configurations, they can be manifolded for flow in parallel to provide relatively high flow rates, low pressure drops and service life to filter several thousand liters of biopharmaceutical process fluids. A complete range of hydrophilic and hydrophobic filter media is available to meet applications in clarification, prefiltration, sterile filtration and virus reduction. The assemblies are available pre-sterilized, to eliminate the need for autoclaving (Fig. 10).

The polymeric—usually polypropylene—housings are provided with vents and drains; gauge ports are available for direct reading of differential pressure. The assemblies are provided with low-holdup designs to conserve valuable biofluids. They can be integrity tested to comply with quality and regulatory requirements. Because the units are designed as single-use and completely disposable, they eliminate the need for cleaning and cleaning validation.

These lightweight assemblies are easily installed wherever they are needed. Larger systems for higher flow rates and larger batch production can be wall-mounted, stand-mounted or skid-mounted. Installation is fast and simple, compared to stainless steel housings.

Applications. Early applications of this new housing technology include filtration of whole serum, tissue and cell culture media, buffers, WFI, microbiological growth media, solvents, acids and bases, sterile and non-sterile venting (Table 3).



FIGURE 9 High-capacity capsule filters are encapsulated cartridge filters available in 4-, 10-, 20-, 30-, and 40-inch lengths. *Source*: Courtesy of Meissner Filtration Products, Inc.



FIGURE 10 High-capacity disposable capsule filter assemblies manifolded in biopharmaceutical production system. *Source*: Courtesy of Meissner Filtration Products, Inc.

BEYOND ULTRA-CAPSULES: DISPOSABLE PROCESSING SYSTEMS

As the biopharmaceutical industry realized, even a disposable plastic cartridge-and-housing assembly with high filtration capacity, singly or manifolded in parallel, had limitations due to its need for peripheral equipment including piping (polymeric or steel), valves, gauges, tanks, carboys or other storage vessels, and filling equipment.

Although they accommodate the need for batch production of biopharmaceutical process and product liquids, and for filtration of process gases, ultra-capsules still operate in batch filtration mode. They must be aseptically installed and carefully handled to avoid contamination or damage, although more robust version of ultra-capsules are now produced to minimize the risks associated with handling.

And while the ultra-capsules are available pre-sterilized and can be autoclaved, the associated tubing or piping, as well as the downstream tanks, carboys or other storage vessels must also be maintained in sterile condition for all sterile applications. If downstream piping, tanks and other hardware are to be re-used, they must be re-sterilized, and their cleaning and sterilization procedures validated.

From these technological and logistical limitations arose the need for completely disposable filter-piping/tubing-biocontainer assemblies. Biocontainers, variously called biobags or bags, have been available for more than twenty years. Plastic film bags, essentially process-scale IV fluid bags similar to those used for decades in hospitals, have been modified to accommodate the volumes of fluids to be processed in biopharmaceutical and biologicals manufacture. Polymeric filter housings, from standard capsules to ultra-capsules, are attached to flexible, polymeric tubing, with clamps and connections, attached to a pre-sterilized polymeric film bag.

TABLE 3 Comparison of Stainless Steel Housings and Disposable High-Capacity Capsules

Design features and operating parameters	Stainless filter holders and housings	Comments	Disposable high-capacity capsule filters	Comments
Cost of 10" housing	$1000-$1500 U.S.	Sharply escalating costs of stainless steel	Price of comparable cartridge filters plus 20-30%	Avoids capital expense small capsule cost premium for benefits of disposability
Flow rates	1 mLpm–1000Lpm		1 mLpm–100Lpm	High-capacity disposable systems manifolded in parallel
Capacity	To 5,000L whole serum		To 5,000L whole serum	High-capacity disposable systems manifolded in parallel
Usage	Reusable	Amortize capital costs—incur higher labor, maintenance and validation costs	Single-use disposable	Use as needed rapid scale-up or scale-down Minimal set-up and clean-up; simple operation
Sterilizable	Steam (housings) or autoclave (both, depending on size)	Inline steaming or autoclaving requires lengthy cooling-down	Presterilized by gamma irradiation	Aseptic handling essential to maintaining sterility; connections, tubing or piping and collection vessels must also be kept sterile
Materials of construction	316L stainless steel		polypropylene	
Temperature limitations	Limited only by filter thermal rating		Polymeric housings, usually PP	PP housings cannot be inline steam sterilized
Surface finish	R_a<20μ-in.		Polymeric	Polymeric surfaces have lower R_a
Filter connections	222, 226 bayonet tab	O-rings can be mis-installed or be damaged in handling or use	Internally sealed	No chance of filter bypass in capsules or ultra-capsules
Housing connections and sizes	TC to 2" hose barb to 3/4"		TC to 2" hose barb to 3/4"	Ultra-capsule connections sanitary flange, hose barb or stepped hose barb or Flaretek®

Cartridges	Retrofit other manufacturers' housings	Range of choices of cartridge or disc filter manufacturer	Preassembled and installed No inter-retrofittability	Integrally-sealed ultra-capsules No need/opportunity for retrofitting
Flow rates	1 mL/min–30 gpm	Depends on fluid viscosity, filter rating, surface area of cartridges or discs, and operating pressure	1 mL/min–30 gpm	Small capsules or disc filters to ultra-capsule systems manifolded in parallel
Throughput	Few mL to multi-1,000L		Few mL to multi-1,000L	
Chemical compatibility	Limited by 316L stainless steel		Limited by filter polymer and PP housing	
Leachables	Validated by manufacturer end-user		Validated by end-user or filter manufacturer	
Extractables	Validated by manufacturer end-user			
Sterilization method(s)	Inline steam or autoclave		Autoclave-gamma irradiation	Autoclave or gamma irradiate
Connection sizes and styles	¼" hose barb to 4" RF flange		3/8" hose barb to 1" sanitary flange	
Cleaning method(s)	CIP, chemicals, detergents, WFI		N/A	

Source: Courtesy of Meissner Filtration Products, Inc.

From this productive amalgam of existing technologies—the capsule and ultra-capsule filter, medical-grade tubing, sterile connections (some innovated in recent years, others using sterile welding techniques previously used in the medical device industry), and derivatives of sterile IV bags—arose the disposable bioprocess container system, variously referred to as "BPC's" (bioprocess containers) or "DPS" (disposable process container) assemblies.

In these assemblies, filters, filter housings, tubing, aseptic connectors, flow control devices, multi-layer plastic film bags ranging in size from <1 to >2000 L liquid capacity, are assembled to constitute a pre-sterilized, disposable bioprocessing system.

Disposable filter housings and related tubing, biocontainer bags, bioreactors and other equipment offer solutions to the industry's needs. Many of the serious shortcomings of stainless steel housings and related hard-plumbed process equipment impede the ability of the biopharmaceutical industry to achieve regulatory compliance, improve manufacturing efficiency and productivity, and decrease capital and operating costs (Fig. 11).

For the unique requirements of the biopharmaceutical industry, the BPC/DPS technology provides a wide range of solutions associated with filter housings, filters, piping, tanks and other storage vessels, and in one technology, sterile filling lines.

In some systems, bioreactors are included. In others, tangential flow filtration is incorporated in addition to, or instead of, membrane microfiltration, depending on whether the system is to be used in upstream or downstream processing operations.

Common biopharmaceutical applications for these filter-tubing-biocontainer/bag systems include:

- cell and tissue culture, microbial fermentation,
- dispensing,
- collecting,
- transporting,
- mixing,
- storage, and
- waste disposal.

Biopharmaceutical firms and CMOs are eager to sustain the highest level of manufacturing excellence and quality, while reducing costs and shortening

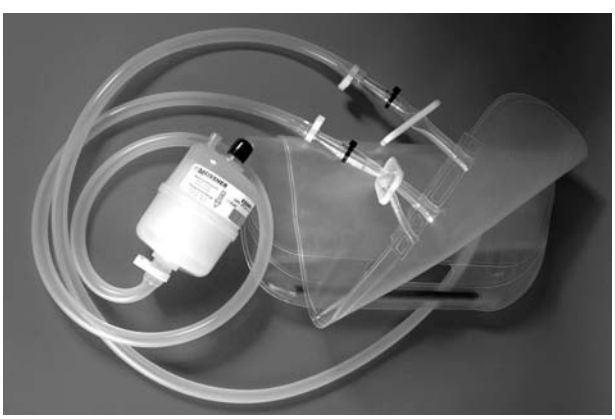


FIGURE 11 Disposable bioprocess system assembly with capsule filter, medical grade flexible tubing and biocontainer. *Source*: Courtesy of Meissner Filtration Products, Inc.

time-to-market for new products. Firms desire to maintain regulatory compliance, while improving production economics.

CMOs have proliferated and expanded apace to meet the burgeoning need for manufacturing capacity. With this growth, CMOs will continue to shoulder a good deal of manufacturing for emerging biopharmaceutical companies, many of which do not have manufacturing capacity for clinical trial quantities of biopharmaceuticals.

CMOs and biopharmaceutical manufacturers need all the technical and business competitiveness they can summon to survive and prosper in this increasingly competitive industry.

CMOs represent the ultimate in need for manufacturing flexibility. Often multi-client manufacturers, they produce multiple products simultaneously in the same facility, and need to minimize manufacturing costs, maximize flexibility in logistics, and be able to change from one product to another as rapidly as possible, to meet customer schedules and maximize their own profits. Disposable ultra-capsules and disposable bioprocessing systems are the optimal solution for CMOs.

DRIVING THE PARADIGM SHIFT

Any paradigm shift in science or technology must be accompanied by improvements and benefits, advantages of the new paradigm over the old. For disposable filter-and-container systems numerous benefits to the biopharmaceutical industry, including both direct manufacturers and CMOs. Relative to stainless filter housings and enclosed filters they:

- reduce or eliminate risk of cross-contamination,
- maximizing yields and minimizing production losses,
- cut capital equipment costs,
- cut design engineering costs,
- shorten construction and installation time,
- reduce building space requirements,
- reduce labor costs of installation,
- improve manufacturing space utilization,
- improve product and batch turnover flexibility,
- increase plant efficiency and profit potential,
- minimize labor costs of cleanup,
- minimize or eliminate cleaning validation requirements,
- reduce or eliminate costs of cleaning chemicals and their disposal,
- reduce or eliminate many of the costs and risks associated with stainless steel housings, including
    - requirements for highly trained, skilled operators,
    - installation,
    - operation,
    - change out,
    - maintenance,
    - sterilization,

- integrity testing,
- validation,
- component replacement,
- filter change outs,
- cleaning,
- cleaning validation,
- integrity testing,
- cross-contamination, and
- out-of-specification (OOS) product.

From the end-user standpoint, adoption of disposable filter-biocontainer systems also provide numerous benefits (Bardo, 2004; De Palma, 2004; Rios, 2003). Some of these are summarized below:

- transition from SS to disposable systems
  - outsources risk, improves economics
- single-use process systems enable
  - faster changeovers in production
  - faster scaleup
  - flexible plant production capacity in plant expansions
  - no CIP or no SIP
  - reduced capital costs
  - reduced labor costs
  - operating cost reductions (materials and labor)
- reduced validation time, labor and materials costs
- shorter time to market
- eliminates cleaning costs and concerns
  - cross-contamination
  - product adulteration—OOS product
- improves plant operating efficiency
- increases plant productivity
- makes plants less expensive to build and operate

These benefits go far beyond the individual user's advantages of using filter-biocontainer systems over traditional stainless steel-and-cartridge filter systems. What is important to understand is that the paradigm shift in filter housings is an essential enabling step in this broader shift to disposability in biomanufacturing. Without this paradigmatic filtration shift, the broader manufacturing change would be difficult or virtually impossible. To be convinced, one needs only to see the warehoused stainless steel filter housings at biopharmaceutical manufacturing companies which have transitioned exclusively to use of disposable filter-biocontainer systems. Stainless steel housings have not disappeared, nor will they from such applications as DI water systems. But, in biopharmaceutical product production, they are a severely endangered species.

Some basic comparisons further highlight the differences between stainless steel and disposable polymeric filtration technologies (Table 4).

TABLE 4 Comparisons of Stainless Steel Housings vs. Disposable Filter-Biocontainer Systems

Design features and operating parameters	Stainless filter holders and housings	Disposable capsule filters-and-biocontainer assemblies	Comments
Flow rate	0.1 Lpm-120 Lpm	0.1 Lpm to 120 Lpm	Smallest disc filters, capsules, to ultra-capsules manifolded in parallel
Capacity			
Usage	Reusable	Single-use, disposable	
Sterilization	Steam (housings) or autoclave (both, depending on size)	Presterilized by gamma irradiation	Separately or as part of disposable processing system with tubing, bag, connections, etc.
Materials of construction	316L stainless steel housing and piping		PP housings—silicone tubing
Surface finish			
Filter surface area capacity (EFA)			
Filter connections	222, 226 bayonet tab	TC, hose barb	
Cartridges	Cartridges with standard connections readily inter-retrofit other manufacturers' housings		Dedicated, pre-sealed, pre-sterilized disposable filter processing systems eliminate need to retrofit
Flow rates	1 mL/min to 30 gpm	Depends on fluid viscosity, filter rating, and surface area of cartridges or discs, operating pressure	1 mL/min to 30 gpm
Throughput	Few mL to multi-1,000L		Few mL to multi-1,000L
Chemical compatibility	Limited by 316L stainless steel		Limited by filter polymer and PP housing
Leachables	Validated by filter manufacturer or end-user		Validated by end-user or filter manufacturer
Extractables	Validated by filter manufacturer or end-user		Validated by end-user or filter manufacturer
Sterilization method(s)	Inline steam or autoclave		Autoclave gamma irradiation
Connection sizes and styles	¼ inch hose barb to 4 inch RF flange		3/8" hose barb to 1" sanitary flange
Cleaning method(s)	CIP, chemicals, detergents, WFI		N/A

Source: Courtesy of Meissner Filtration Products, Inc.

CONCLUSION

The evolution of filter housings for the biopharmaceutical industry has seen a great diminution in the use of stainless steel filter housings, which are being displaced by a variety of single-use filter-bioprocess container systems.

The paradigm shift in filter housings from stainless to polymeric is part of a wider shift from hard-plumbed biopharmaceutical manufacturing plants to disposables-based manufacturing processes and plants. The argument can justifiably be made that the paradigm shift in filter housings has served to enable the wider change in the industry. The move to disposable filter housings and filter-bioprocess container systems is an essential building-block of the fast-developing change toward disposability and more flexible operations in the biopharmaceutical industry.

While many technologies have served to enable the paradigm shift in biomanufacturing, a necessary, if not sufficient, requirement is the increasing availability of reliable, safe, disposable polymeric filters and biocontainers in a wide range of liquid capacities.

The filter housing paradigm shift has been an essential prerequisite to the wider biopharmaceutical manufacturing paradigm shift.

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9

Stainless Steel Application and Fabrication in the Biotech Industry

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INTRODUCTION

When viewed against the scale of world history, both biopharmaceuticals and stainless steel are virtual newborns. Notwithstanding, however, since its discovery little more than a 100 years ago, stainless steel has become a significant material choice for numerous and varied processing industries including food, beverage, drugs, and cosmetics, yet these represent only the tip-of-the-iceberg when one considers the breath of its application range in medical devices, power generation, automotive systems, architecture, maritime use, chemicals, kitchen appliances, barbecues, furniture, transportation, and aerospace.

Within this chapter, a brief history of the pharmaceutical industry will partially parallel the development of stainless steel alloys to provide background and to allow for an understanding of their convergence. In addition, a basic explanation of stainless steel metallurgy, corrosion resistance, and application will be provided prior to discussion of fabrication and polishing.

Since the breadth and depth of this subject cannot be fully explored within the confines of a small section of this text, only general summary information will be offered primarily as a companion to the other materials which are the real subject matter of this volume. Much of this information has been simplified for that reason, and there may be instances where this summarization will result in explanations that will be less than fully correct from a rigorous scientific or technical perspective.

A BRIEF HISTORY OF PHARMACEUTICAL PHARMACEUTICALS

The recorded history of pharmaceutical products dates to at least as far back as 3000 BC based on the discovery of Sumerian tablets from that era inscribed with human prescriptions. Over the course of the ensuing 5000 years, events throughout the centuries crisscross the globe from Baghdad to New York City as the efficacy, quality, and safety of pharmaceutical products evolve, struggling through ethical and developmental crises, constantly improving both the duration and quality of life we enjoy today and expect to enjoy even more fully in the future. Often, the process has been painfully slow with the vast majority of significant improvement in drug discovery, development, processing, and delivery occurring during the last 100 years. The student of pharmaceutical history will

find more than ample material for further study in topics such as the Magna Carta of Pharmacy, the first Pharmacopeia, Germ Theory, Paracelsus and Variolation, as well as a plethora of other events, individuals, and activities that have ever so painstakingly moved the pharmaceutical industry from a black art to a serious and critical science. Those with an interest can easily find substantial data to detail this development, including especially important ethical and legal milestones.

During ancient times, as might be expected, drugs and their ingredients were natural substances obtained from plants, animals, and the earth. They were stored in what were then suitable and appropriate containers, including earthenware vessels, animal skin pouches, and bone, rock, or wood receptacles fabricated using implements that we would undoubtedly consider extremely crude, by today's standards. Certainly, during those times, the interaction between the ingredients and their containers was of little or no concern and undoubtedly the mere ability to enclose and somewhat protect these materials was their most significant benefit. Thankfully, as time unfolded, humankind developed tools, materials, and techniques that would spur further advancement, and is reflected in the birth of modern chemistry, metallurgy, manufacturing, and medicine.

Not surprisingly however, the field of medicine has often lagged behind many of the other areas of science. This was especially evident during wartime periods when developments in transportation, munitions, and armament were put to use, far exceeding the ability of physicians and pharmacists to care for those surviving the carnage. Of note, there were less than 24 effective drugs known prior to 1700, and most of those were plant based including such items as aloe, figs, and senna. Additionally, alcohol predominated in most early remedies obviously because of its numbing and/or euphoric effects. During the ensuing post-Sumerian millennia one might have expected to see significant improvement in pharmaceutical products and medical technology, however as late as 1878 it was still relatively common for prescriptions to have little or no scientific merit, such as this script written by Dr. H.C. Wood for peritonitis (Dowling, 1990), an inflammation of the stomach lining.

Dr. H.C. Wood-1878
prescription for peritonitis

- Bleed patient until faint.
- Apply leeches to the abdomen.
- Multiple doses of calomel (laxative).
- Render unconscious with opium.

During much of medical history, societies endured in spite of the treatments and their practitioners. Medical care was often the purview of witchdoctors, priests, minstrels, traveling salesmen, carnies, and other charlatans making healthcare a source of concern, uncertainty and most often, fear for the patient. Drugs, primarily from plant and animal sources, were variable in their effectiveness at best and in the hands of the unscrupulous, ranged from ineffectual to lethal. Neither practitioners nor patients understood drug activity within the body and the study of pharmacokinetics and pharmacodynamics had yet to come into existence. Fortunately, over time, advances in materials allowed for somewhat improved formulation and storage of products and ingredients. Elegant constructs such as glass and ceramic were pressed into service, although certain more basic and traditional materials remained prevalent. As we approached the twentieth century, in a practical sense we had moved significantly

ahead, but in a real sense, our medicines and understanding remained primitive. By this time, drug product development had become the realm of the chemist and pharmacist, although processes had been improved and refined, a huge gap remained between the chemistry capable of producing active entities and our ability to formulate, dose, and safeguard patients.

Many of the issues faced in the drug arena were also stumbling blocks for the food and beverage industries, and it may be surmised that similar challenges combined with FDA as a common regulatory base, led to parallel solutions and the ability to apply technology across marketplaces. One of the most significant and defining moments for pharmaceuticals came in large part as a result of events involving the food processing industry, based on the publication of *The Jungle* by Upton Sinclair in 1906. Unsanitary and dangerous conditions in the Chicago meat packing industry were exposed by Sinclair, and when word of these problems was made public, and in light of other known and serious cases of food and drug adulteration, enactment of the Pure Food and Drug Act under then President Teddy Roosevelt was inevitable.

During the same general historical timeframe, humankind's development of implements advanced from Stone Age to Bronze Age and eventually to the modern era. During the eighteenth and nineteenth centuries, the use of iron and the development of steel allowed for the fabrication of machinery and tools, however, these materials were susceptible to atmospheric corrosion and had limited application in wet environments especially when salts were present. Hence, as early as 1821, scientists and metallurgists, recognizing the need for non-corroding metals especially for cutlery, began experimentation using chromium to enhance the properties of iron and steel. Dozens of alloys were created, throughout much of that century, ranging from Ferrochromium to numerous types of steel containing varying amounts of chrome, nickel, carbon, and other alloying materials. Unfortunately, much of the early work missed its mark based on crude investigations, weak research, and bad luck. The most significant issues related to the failure to properly understand the interaction between alloying substances and development of appropriate test methodology. Finally, during the late 1800s scientists began to recognize these gaps in exploring mid-range alloy formulations and the relationships between alloying materials. As a result, early forms of the family of alloys we today call "stainless steels" began to emerge.

Interestingly, as we began the twentieth century, glass, ceramic, tin, and other fairly common materials continued to be mainstays in drug manufacture while differing "schools of thought" began to emerge in other industries, especially food processing. In spite of the relative inertness of glass and ceramics, these materials were perceived, albeit inappropriately, to be weak and susceptible to breakage. This, coupled with their limited options for fabrication, opened the door to other materials better suited to market requirements wherein dairies and food processing plants were among the earliest users of stainless steels.

STAINLESS STEEL DEVELOPMENT

Stainless steel itself is not an alloy, but rather it is a family or group of alloys based on the addition of greater than 10–12% chromium to iron or steel which imparts resistance to chemical attack similar to noble metals such as gold (Zapffe, 1949:1).

The quest to develop stainless steels was not based on a burning need within the healthcare industry but was based on the cost of corrosion of iron and steel which had been estimated to average approximately $3.5 billion annually between 1900 and 1950

(Zapffe, 1949:5). It was that cost that motivated scientists and metallurgists to seek an alloy that would not rust or corrode under normal atmospheric conditions. Little did they know how successful they would be once the alloying puzzle was completed, "enabling a new era in engineering" (Zapffe, 1949:2), that would go far beyond the basic corrosion resistant features of stainless steel.

Initial work begun by Bertier in 1821 resulted in the creation of Ferrochromium, but it would take another 100 years before the primary grades of stainless would be determined and their characters understood. During this period, dozens contributed significantly including such prominent names as Guillet, Monnartz, Giesen, Brearley, Portevin, Maurer, Strauss, Becket, Dantsizen, Field, and Monypenny. When considering stainless steels of any type, the underlying principle that must be recognized is passivity, for it is passivity that gives stainless steel its stainlessness and corrosion resistance. Passivity is ultimately based on a chromium enriched (iron depleted) oxide film that occurs on the surface of the metal. Chrome/Iron ratios in the range of 0.8–1.2 are fairly common, with higher ratios more desirable. Passivity will be discussed in further detail in subsequent pages; however it will remain the most important underpinning for the application and use of stainless steels.

The discovery of stainless steels and those responsible has been chronicled by numerous authors, however it is appropriate to make specific note of the text, *Stainless Steels* written by Carl A. Zapffe (1949), that this author has used extensively as a reference. Zapffe makes note of the discovery of the constitution, corrosion resistance, and industrial usefulness of stainless steels including assignation of those individuals that were most responsible. *Stainless Steels* remains an extremely valuable reference volume in spite of its 1949 date of publication and it is well worth the effort to locate and obtain a copy.

Stainless steels are often classified by their structure using roman numerals, and Zapffe identifies three classes. Class I stainless steels are referred to as "martensitic stainless steels" because martensite characterizes their microstructure when hardened. This particular feature also gives rise to alternative nomenclature such as "hardening" or "hardenable" stainless steels, typically by way of heat treatment. Class II alloys are characterized by an extremely low carbon content resulting in an iron-chrome alloy more appropriately defined as a type of iron than as a type of steel. Notwithstanding, this group is designated "ferritic stainless steel," again based on its microstructure, comprised primarily of ferrite. Finally, Class III alloys include the grade commonly referred to as 18–8 stainless steels with approximately 18% chromium and 8% nickel. These grades are designated "austenitic stainless steels" with a microstructure based on "austenite."

Details of the exact structure, formation, phase transition, and sub-phases for each of the classes are beyond the scope of this text. Extensive research has been performed in these areas and an abundance of material is available for anyone interested in expanding their knowledge base. Class I stainless steel usually includes chromium in the range of 12–17%, while Class II grades have chromium between 18% and 30%. Class III alloys include chromium in the range of 8–30%, however this group introduces nickel as another major alloying material and may also include molybdenum as well as other lesser used materials. Microstructure designations will be reviewed more fully on subsequent pages along with additional detail, while remaining true to the simplifications applied thus far.

Passivity

Returning to the subject of passivity, it should be made clear that passivity is a condition of negligible corrosion rather than non-corrosion, and passivity is relative

since material that is passive with regard to a particular medium may not be passive, or may corrode, in another. The passive layer, or film, that protects the stainless steel is extremely thin, estimated on the order of 5–50 Å (1Å=1 ten billionth of a meter), yet is capable of endowing the material with corrosion resistance near that of noble metals such as gold, silver, and platinum. This passive film occurs when the chromium content of the steel nears 12% and improves to some extent with increasing chromium levels as well as with the addition of nickel and possibly other substances to the alloy. It should also be noted that damage to a passive surface results in depassivation, or activation, of the material allowing chemical attack to occur. Hence in dynamic applications, such as those within a pharmaceutical environment, passivity must be monitored and periodically repassivation must occur to ensure a stable surface condition remains. It is well recognized that passivity is naturally occurring in air; however the rate and degree of passivation may be inadequate for a particular application depending on individualized circumstances. Materials which have had adequate time to achieve passivity after fabrication will typically achieve a relatively high degree of passivity, however, naturally occurring passivity is still usually less robust than passivity artificially induced via a deliberate and methodical treatment process. Materials that have recently undergone fabrication of one type or another that disturbed the passive layer will most likely require repassivation prior to being put into service, especially if the period prior to use is small or if the material might be inhibited in any way from achieving natural passivity. Artificially induced passivity is usually implemented via a multi-step process that includes a cleaning of the material surface to remove any grease, oil, or other contaminant that might interfere with passivation, typically using a mild caustic cleaner. The cleaning is followed by a passivation step whereby an acid such as nitric, phosphoric, or citric, occasionally with additives such as chelants, contacts the metal surface expediting its passivation. Rinsing steps will most likely occur at various points in the process using water, keeping in mind that the quality of both the chemicals and the water must be appropriate for the application to avoid introduction of contaminants.

Passivation can be verified using an indirect test such as ferroxyl (copper sulfate) as well as via electro-chemical spot tests, readily in the field. Alternatively, direct tests such as auger electron spectroscopy (AES) and X-ray photoelectron spectroscopy (XPS) are also available for use, however, both are significantly more expensive, may require sample destruction and are more difficult to implement. Since artificially induced, or assisted, passivation involves the handling and disposal of dangerous chemicals that may contain heavy metals, it is usually recommended that only firms with proper expertise perform the procedures, especially if they are required in a field setting rather than in a controlled manufacturing environment.

Preoperational passivation is a common requirement in the pharmaceutical and biotech industries and is accomplished in a relatively easy fashion as the metal has not been degraded, as yet, in any way by product contact. Repassivation is often complicated by the presence of rouge on the surface of the stainless steel, which must be removed before any attempt at repassivation should be made, as the rouge will typically not allow the required intimate contact between the passivating chemicals and the metal surface. Passivity cannot be overemphasized as it is the singular primary reason that stainless steels exhibit their corrosion resistance. Incorrect alloying based on too little chromium, too much carbon, or improper levels of other constituents will result in a material unable to form a suitable film and not qualified to function as, or be termed, stainless steel. If insufficiently passive, any stainless steel will be subject to corrosion in varying degrees based on

the condition of the surface film, the corrosive media, and other important factors such as temperature.

Corrosion

The corrosion resistance of stainless steel, as aforementioned, is based primarily on the passive film or coating that develops on the surface of the metal, hence retardation, destruction, or removal of the passive layer renders the surface non-passive or active, and subject to corrosive attack. Although there were many who shared in the honor of discovering stainless steel, four names stand out as the most significant including Giesen, Guillet, Monnartz, and Portevin. Within that small group, it was Monnartz (Zapffe, 1949:15) whose writings in 1911 provided key insight into the nature of stainless steel by discovering that: (a) the corrosion resistance of 12% chromium steels increased dramatically; (b) passivity was responsible for the improved corrosion resistance; (c) passivity depended upon oxidation of the surface metal; (d) pre-operational passivation improved performance in corrosive environments; (e) a relationship existed between passivity and the temperature of a corrosive environment; (f) formation of carbide precipitates retarded or prevented passivity; (g) carbon stabilized with other alloying elements such as molybdenum can be kept from interfering with corrosion resistance; and (h) addition of molybdenum had an especially favorable effect in enhancing corrosion resistance.

Stress corrosion cracking or environmentally assisted cracking of stainless steel can result in catastrophic failure and should obviously be avoided at all cost. This failure is typically the result of interaction between stress, environment, and microstructure. Failures of this type often involve chlorides and have occurred in the vicinity of swimming pools and as a result of chloride containing insulation on stainless steel tanks and piping. Insulation related failures have caused the shutdown of nuclear reactors and resulted in the rupture of tanks containing hot USP water, causing serious personal injury to operations personnel.

Generally accepted theory today holds that pit corrosion may be the most common form of corrosive attack on stainless steel in pharmaceutical product contact applications. This type of corrosion is typically more subtle beginning with a surface defect or fault in the passive layer. As a result, the underlying metal begins to dissolve leading to a build up of positively charged metal ions. This in turn attracts negatively charged ions, most likely chlorides, to the vicinity, causing the local pH to drop to as low as 2–3, even in a neutral solution, and retards reformation of the passive film.

Pit initiation is not well understood, however, it is thought by Ryan et al. (Bhadeshia and Sourmail, 2005) that manganese sulfide (MnS) inclusions, difficult to avoid in the manufacturing process, coincide with chromium depletion at the pit boundary. Pitting indexes can be calculated for the various grades of stainless steel and should be considered when making application selections.

Stainless alloys can become sensitized, and the resultant sensitization can allow stainless steel to undergo changes resulting in decreased corrosion resistance. The change that occurs is the formation and precipitation of chromium-rich carbides. The resulting chromium depletion at the grain boundaries results, even under stress-free conditions, in anodic intergranular attack.

Rouge

Rouge is an iron based contamination on the surface of the stainless steel occurring most notably in aqueous environments, in varying colors, textures, and levels of adherence.

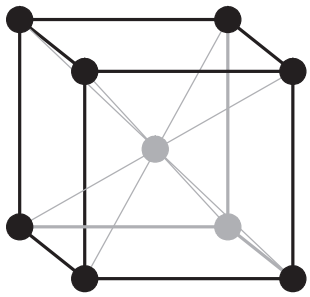
The amount and degree of rouging is commonly based on temperature with higher temperatures resulting in darker colors and significantly stronger adherence. Rouge, in its initial stages, is typically orange or reddish-brown in color and loosely attached, often with a powdery look, which might be removed using only a soft cloth. As rouge continues to develop, colors darken and surface bonding increases such that removal using chemical dissolution is required. In its worst stages, often found in clean steam systems, rouge is black in color and adhered so tightly that the aggression required to remove it will most probably damage the material surface significantly so as to require re-polishing, if thinning has not progressed too far, or even replacement. Although research into the details of the rouging phenomenon is incomplete, it is believed that once rouge has adhered to the stainless surface pitting eventually commences, depassivating and activating the surface of the metal, allowing further corrosion to occur. Hence it is accepted practice to periodically derouge and then repassivate the in-service metal initially based upon visual inspection techniques, and subsequently by extrapolation of historical data to determine a reliable and predictable schedule, as rouge formation is not usually erratic. Rouge removal may not be consistent from application to application or system to system, making it prudent to construct systems with removable coupons that can be tested off-line for the purpose of determining a completely reliable removal procedure for a specific situation.

In spite of its unappealing look, rouge seldom causes purified water quality degradation and often only causes contamination of the products it contacts when it sloughs off of the stainless steel surface as undesirable particulate, and as is obvious, in all cases foreign particulate is undesirable and unacceptable in pharmaceutical and biotech products. Tverberg (1998) has written extensively on rouge and metallurgical aspects of stainless steel and is an excellent reference for additional insight into these alloys as well as expanded understanding of the degrees, implications, and nature of stainless steel rouge, based on his in-depth research and substantial industrial experience.

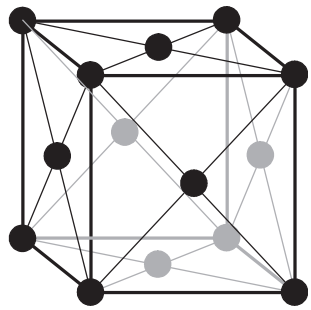
STAINLESS STEEL CLASSIFICATION

Returning to the discussion of microstructure, the division of stainless steels by class results in delineation of the primary types along with their basic characteristics, however it should be noted that within each class Zapffe (1949:115) defines subclasses (typically three for each) which further differentiate the alloys within each group. Sequential lettering added to the numeral designation as in Classes IIIa, IIIb, IIIc allows for the further classification as work hardenable, free spinning, and modified, respectively. It is also important to note that many of the categorizations used are generalizations which cannot be absolutely applied, but rather are provided for simplification purposes and to aid general understanding. As well, more current classifications alter slightly this subclass structure as will be mentioned in the following pages.

The terms ferrite, martensite, and austenite, previously mentioned, refer to the microstructure of the metal. Iron does not occur environmentally in its pure form because of its tendency to combine with other elements such as oxygen. Once extracted from its ore, if unprotected, iron will rapidly react in a reversion process, such as rusting, to its prepurified form. Iron, in its pure form can exist with either a ferritic crystalline structure, depicted as a body-centered cubic atomic model, or as austenite represented by a face-centered cubic atomic model.



Body-centered cubic model



Face-centered cubic model

This allotropic character results in many technical nuances related to the atomic density, however these issues are beyond the scope of this discussion. The third structure mentioned, martensite, can only occur in impure or alloyed iron and is "actually a distorted and unstable arrangement of atoms caught in transition from austenite to ferrite" (Zapffe, 1949:86). However, it is the very character of martensite that allows for hardening by heat treatment and its designated uses.

The family of alloys known as stainless steel is commonly depicted using a tree as the model. The basis for this appears to be with Zapffe, since *Stainless Steels*, published in 1949, begins with this artwork and includes no acknowledgement. However, it is interesting that many subsequent writers also utilize the "tree model" without assignment of credit to Zapffe or others.

In his graphic of the tree, Zapffe shows a simple trunk and limb structure emblazoned with the various classifications of stainless steel. The simplicity of the tree concept is superb for visualizing how the different classes and grades of stainless steel increase with the height of the tree, along with increasing chromium and cost. Without the benefit of hindsight, and recognizing the infancy of the industry, Zapffe refers to the limbs as branches hence the following explanations will continue that reference, at least in the short term.

Class I stainless steels as mentioned previously, are primarily martensite. The members of this class are hardenable via heat treatment, with corrosion resistance higher in the hardened forms, and as a result they are very well suited to uses such as surgical instruments, knives, fasteners, turbine parts, bearings, shafts, and springs. Also called "straight chromium" stainless steels, they exhibit lower corrosion resistance based on the chromium (12–17%) and carbon (< 1% typically) constituent levels with few grades having other significant alloying materials. A number of the 400 series stainless steels are considered Class I including 403, 410, 414, 416, 420, 431, and 440. Class I members are usually magnetic and can be difficult to weld or fabricate while maintaining their martensitic structure. It is also worth noting that most Class I stainless steels require stress-relieving in their fully hardened condition, and that stress-relieving via heating will, of necessity, occur below the temperatures which might modify the martensitic structure of the material. Also, Class I stainless is often sensitive to enbrittlement from hydrogen gas, particularly in the hardened condition. This can occur from moisture in the manufacturing process or from pickling solutions used in post-treatment. Class I stainless is represented as the "first branch" of Zapffe's tree although it is more appropriately described as the trunk, rooted in the ground where it connects to its iron source.

Class II stainless steels are typically referred to as ferritic or non-hardenable stainless steels based on the primarily ferrite crystalline structure that exists in the

metal. These 400 series grades, including 405, 409, 430, 442, 443, and 446 contain chromium in the range of 12–27% with carbon usually below 0.2% (except for 446 with 0.35% carbon maximum). These alloy variants are magnetic, cannot be hardened by heat treatment, although some can be work hardened, and are also difficult to weld. Applications vary widely including uses such as cooking utensils, architectural trim, chemical processing equipment, heating elements, automotive exhausts, fasteners, bank vaults, decking, and household appliances. Alloying substances beyond chromium are used, including aluminum, copper, tungsten, and silicon for benefits including improved weldability, greater hot strength, and higher oxidation resistance. These are the second branch of the Zapffe tree, with both having higher corrosion resistance and higher cost, as depicted by their elevation on the tree.

The third and final branch of the Zapffe tree is the Class III alloys which are austenitic in structure. This group of materials includes 300 series stainless steels and utilizes additional alloying materials, most importantly nickel and molybdenum, for increased corrosion resistance and improved workability.

Austenitic alloys are typically non-magnetic in the annealed condition and cannot be hardened by heat treatment although hardening by cold working is common. These grades, highest on the tree, represent the best corrosion resistance (especially 310, 316, and 317 grades) and have the added benefit of being easily welded. In addition, excellent cleanability and exceptional resistance to both high and low temperatures make these stainless steels well suited for application in food processing, kitchens/restaurants, architecture, pharmaceutical/biopharmaceutical manufacture, chemical processing, ovens, heat exchangers, marine applications, and hospitals. Of note, the substantial amount of 18-8 stainless steel designed into the Art Deco ornamental top of the Chrysler building in New York City by William Van Alen and completed in 1930 remains in excellent condition after many decades exhibiting remarkably little corrosion. Grades comprising Class III include 302, 304, 308, 310, 316, 317, 321, and 347 that typically fall into the 18/8 category with 18% chromium and 8% nickel. Grades such as 316 also include molybdenum represented albeit incorrectly as 18/8/3, for increased resistance to chloride corrosion. Carbon for these grades is kept typically in the range of 0.08–0.20% although low carbon grades with 0.02–0.03% maximum, discussed later in more detail, have grown in use significantly.

Sensitization, one potential weakness of these grades, should be recognized because of the potential formation of carbide precipitates and will be discussed again relative to welding and fabrication. As might be expected, the alloys in this class represent the more expensive of the basic grades of stainless steel. As noted earlier, the depiction of stainless steel as a tree has continued extensively and as the industry has grown, so has the tree. Today, the primary tree remains as generally described by Zapffe, however the original three classes are now better classified as limbs, rather than branches, and new limbs have "grown" and from these limbs new branches have also been added. This can be seen in Figure 1, reproduced with the kind permission of the Specialty Steel Industry of North America (SSINA), as it appears as part of their Stainless Steel Information Center and website (http://www.ssina.com).

This revised tree includes a fourth branch (limb) representing Duplex Stainless Steels, reclassified (from modified) since Zapffe's time. These grades, such as 2205, are comprised of 18–26% chromium and 4–7% nickel, with a resulting crystalline structure that is a combination of ferrite and austenite, hence the name "duplex." In addition, duplex grades generally have a higher resistance to stress corrosion and chloride attack making them more suitable than the 300 series for application with sea water, pickling,

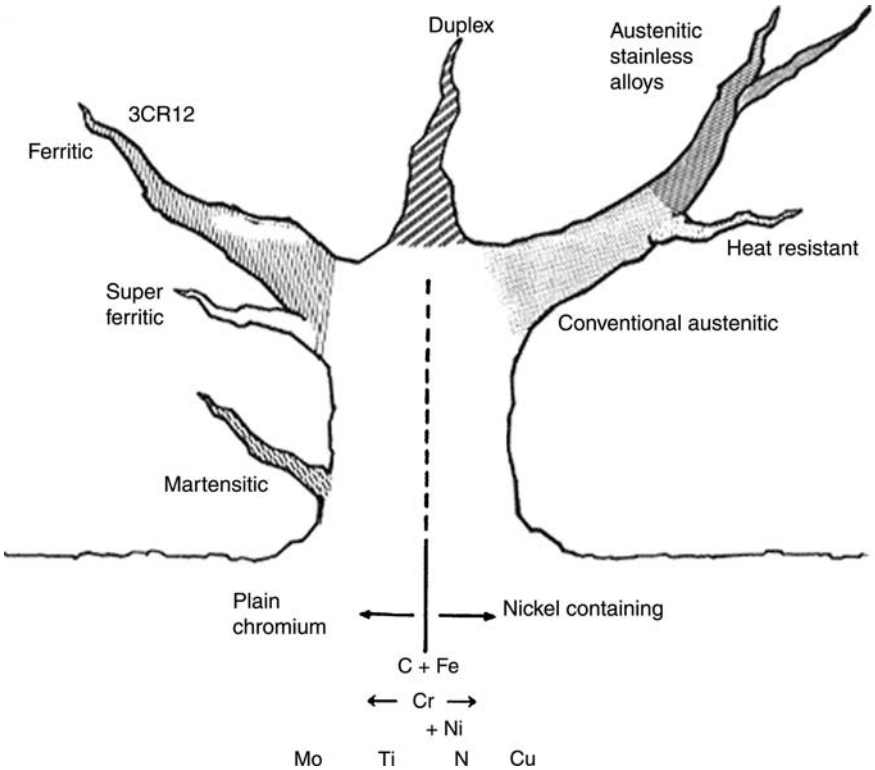


FIGURE 1 The family tree of stainless steel. *Source*: Courtesy of the Specialty Steel Industry of North America.

and desalination operations. Duplex stainless formulations exhibit excellent weldability and have the added benefit of increased strength, although relatively expensive.

The SSINA revised tree also depicts new branches representing development of "super" grades of ferritic, duplex, and austenitic stainless steel including alloys such as AL6XN shown in Table 1 as compared to 304, 304L, 316, and 316L stainless steels.

APPLICATION AND WELDING OF STAINLESS STEEL

As noted previously, the food and beverage industries quickly adopted stainless steel for use in processing because of its corrosion resistance, especially considering the heavy added stress imposed by routine cleaning regimens. Based on relatively low price and

TABLE 1 Comparison by Major Elements

Material grade	Percent chromium	Percent nickel	Percent carbon	Percent iron	Percent molybdenum
304SS	18–20	8–12	0.08 max.	65–71	0
304LSS	18–20	8–12	0.03 max.	65–71	0
316SS	16–18	10–14	0.08 max.	62–69	2–3
316LSS	16–18	10–14	0.03 max.	62–69	2–3
AL6XN	20–21	23–25	0.02 max.	46–51	6–7

ease of fabrication, 304 stainless became the predominant material for use in these applications with vendors specializing in all manner of sanitary components that evolved to meet the demand of a constantly increasing population. In parallel with developing materials technology was the genesis of sanitization and sanitary design. The ability to clean and maintain stainless steel without the fear of corrosion coupled with a better understanding of microbiology and sanitation allowed for equipment and component design that would result in a quality revolution. Organizations such as the International Association of Milk, Food and Environmental Sanitarians, then a part of the U.S. Public Health Service, developed a series of accepted standards and practices designated as "3A" to ensure the quality, consistency, and cleanability of equipment used in food and dairy processing. This drive toward cleanability and sanitary design ensured that the developing industry would take full advantage of the properties of stainless steel especially during fabrication and finishing. It is prudent to mention, at this juncture, that the use of series 304 stainless steel was eventually transferred to the pharmaceutical industry along with the related sanitary technology, however 304SS was considered inadequate for the tougher pharma environment. Eventually the pharmaceutical industry standardized on the use of 316SS, and later 316LSS, for virtually all stainless applications. This may have occurred in part based on the use of stainless steel for high purity pharmaceutical water and steam systems, operating at or above 185˚F (85˚C) temperature, since water in these circumstances can become very aggressive.

The actual manufacture of stainless steel, often in an electric arc furnace, is beyond the scope of this discourse, however the resulting ingots, billets, or slabs are typically produced and used subsequently, via forging or hot rolling, to form various standard sizes and shapes, of rod, bar, sheet, and plate. It is from these forms that most secondary fabrications are made. For example, in the case of a processing vessel 8 feet (96 inches) in diameter with 8 foot (96 inch) sidewall, multiple standard size sheets of stainless would most likely be welded together to form the body, or shell of the vessel. Assuming for this example standard sheets of 4 feet × 8 feet (32 square feet), more than six sheets would be required to form the body, and as well, the top and bottom of the vessel might also require fabrication from more than one sheet. It should be noted that sheet and plate differ typically in their thickness and depending upon the application, plate could be substituted for sheet in this example, if operating pressure so dictated.

Another example is the stainless steel tube and pipe used throughout pharmaceutical and biopharmaceutical processing plants as structural members, to transfer ingredients, finished product, and utilities, and even to serve as conduit for electrical wires. The majority of this material is fabricated from flat stock of appropriate thickness that has been cut to the correct width and is often coiled. This strip material is then processed through a set of dies that form it into the proper shape prior to longitudinal welding to create the finished tubular product. Tubing, 1.5-inch and larger, is typically made in this fashion based on cost, however tubing below 1.5-inch may also be welded or may be seamless based on economics. Large diameter (>1 inch) seamless tubing is available in very limited supply and seldom used due to the extremely high cost on a per-foot basis. It should be noted that tubing is typically defined by its outer diameter such that 2 inch tubing measures exactly 2 inch OD, whereas pipe is typically defined by its interior diameter making 2 inch pipe only nominally 2 inch on the ID. As well, tube wall is most often referred to by gauge, such as 16 gauge (measuring 0.065 inch for 2 inch OD size) whereas pipe is typically produced in schedules such as Schedule 5 (measuring 0.065 inch for 2 inch Schedule 5 pipe size).

Since the tolerances for tubing are frequently more stringent, tubing has become the standard for polished sanitary materials, as control of wall thickness can be better

achieved. Specifications that provide for dimensional tolerances of both pipe and tube were developed by the American Society for Testing Materials (ASTM) including standards such as ASTM-A312, ASTM-A269, and ASTM-A270, the later of which defines specific tolerances for tube that will be polished, primarily for sanitary application.

Another important aspect of sanitary materials is the method by which fittings and accessories are created. Elbows used to construct transfer systems are made from the tubing described above that has been cut into sections specifically for that purpose and then bent appropriately to create the proper shape. Tees that allow for connection of instruments and which also serve to split flow are also manufactured from the same tubing. Cut sections are placed into a fixture and a hole is drilled in the side. A forming sphere of the correct size is then pulled through the hole creating a collar on which an extension is welded. This method is less expensive and provides a higher quality than alternatives such as saddle welding to create the same type tee fitting. Additional types of fittings such as reducers, adapters, caps and sanitary flanges (ferrules) are manufactured by various techniques including machining, forming, and in the case of clamps used to join two sanitary flanges; forging.

It should also be noted that the requirements for dimensional accuracy of sanitary materials goes beyond that needed simply for polishing. Sanitary fittings are specially designed to create a cleanable interior surface. Sanitary flanges are constructed to align precisely when assembled to minimize any hold-up of product or cleaning chemical, as even minute amounts can result in bacterial proliferation, cross contamination, or contamination byproducts that might adulterate subsequent batches. It is for this reason that standard threaded connections, flanges, and other non-sanitary joining components and methods are unacceptable in virtually all pharmaceutical product contact applications. Sanitary fittings must be dimensionally accurate so that they also align perfectly for welding, especially with automated equipment, which will be discussed later, for the same reasons as when assembling via clamps and gaskets. Gaskets for sanitary applications are specially designed to ensure proper alignment and minimal gasket intrusion into the product area, providing proper torque is applied. Notwithstanding, sanitary fittings for assembly of components should be used sparingly and appropriately to facilitate disassembly for service, repair, and replacement. Even though sanitary fittings are designed to provide a high level of cleanability and reliability, their use should be weighed carefully against the use of welding and applied only as needed to suit the application since welding is far superior in joint integrity without the requirements for gasket replacement and retightening of clamps on a continuing life-cycle basis.

Polishing of stainless steel is another critical aspect of producing high quality sanitary materials. Polishing can be accomplished either mechanically, using abrasives or electrochemically in a process called electro-polishing, although electro-polishing is usually applied over a high quality mechanical finish. Polishing will be discussed in further detail in subsequent sections.

Stainless steels can be joined by most conventional techniques including welding, brazing, and soldering, however welding provides the highest quality and strongest joint especially when polishing is required. Welding is the only practical way to permanently join multiple pieces of stainless steel together so that they act as a single piece. Welding as defined by the American Welding Society (AWS) and quoting Cary, is “a localized coalescence of metals or nonmetals produced either by heating the materials to suitable temperature with or without the application of pressure or by the application of pressure alone with or without the use of filler material” (Cary, 1979:21).

Welding using the Oxy–Fuel Gas process is typically the only fuel gas process utilized and is limited to sheet typically not thicker than 3 mm. This limitation is based on slower heating and the large area affected by the flame. Other techniques often utilized are electrically driven and include Shielded Metal Arc Welding (SMAW) and Gas Tungsten Arc Welding (GTAW), however it should be noted that no less than 12 alternative methods are also in use, based on the material grade, thickness, desired speed, and quality requirements. Electrically driven welding can be configured as straight polarity where the electrode is negative, focusing maximum heat at the electrode or reverse polarity with the electrode positive, focusing maximum heat at the workpiece. As noted, many welding processes may be capable of performing the work however, the determination of which welding process is most suitable for an application may be driven by cost or quality considerations. For most commercial applications, speed translates to lower cost with value measured by "deposition rates" based on the amount of filler metal deposited during the welding process. Yet it may surprise those unfamiliar with the breath of the welding industry that 500,000 tons of filler materials were sold along with $1.2 billion in welding equipment during 1976 alone (Cary, 1979:16–17). SMAW is also termed "stick" welding and involves a coated consumable electrode, the core of which becomes part of the finished weld. The coating on the outside of the "stick" protects the molten metal from the atmosphere and results in slag formation that is typically chipped away after the weld is completed. The welder wears a helmet (also called a shield) with a filtered lens that provides protection for the face, neck and eyes from heat, radiation, and spatter that would cause injury.

The other process mentioned is GTAW (or just GTA) called alternatively, tungsten Inert Gas Welding (TIG) that utilizes a nonconsumable tungsten electrode and inert gas for shielding in lieu of the coating used with SMAW. The electrodes are usually alloys themselves with materials such as thorium and cesium added to increase their effectiveness. These radioactive additives can cause safety concerns and must not be overlooked especially where large volumes of the material might be stored. Tungsten, with the highest melting point of all metals, at 6170˚F (3,410˚C), is ideally suited for service as a welding electrode. Electrodes for manual welding are typically ground to a sharp point to create a small precisely focused arc and their length is of minimal concern since the arc gap is controlled by the welder. Alternatively, for most sanitary orbital welding, dimensional control of the electrode is more critical. Arc gap is a function of electrode length hence precise control is necessary for proper welding and required by the equipment. In addition the tip is ground parallel to its length and a flat is added in lieu of a point. These modifications serve to direct and control the arc more fully. The inert gases utilized are typically argon or helium (sometimes also referred to as Heli-Arc), however other gases such as nitrogen, even though not inert, are occasionally used for specialized applications. Argon predominates in sanitary stainless applications and can also be applied as a mixture with helium or hydrogen. These mixture gases provide the attraction of reduced heat input required to accomplish the same function as would be accomplished with only pure argon with higher heat. In addition, the purity of welding gases can impact the quality of the finished weld. Industrial grades of gas, termed "pre-purified" may have purity levels of 99.996% however it is not uncommon for levels 99.998% or higher to be specified. Shielding gases can be supplied either in liquid form or as a compressed gas. Containers or cylinders used for compressed gas are typically smaller than those designed for liquid materials making justification of liquid more difficult for smaller projects even though liquid materials typically offer higher purity levels and are less costly per cubic foot. GTA/TIG has numerous advantages when compared to SMAW as it allows for more precise control, a cleaner weld exterior, and the flexibility to use filler material or to

simply fuse the parts together. There are also advantages relative to automation as well. The welding processes discussed above are traditionally manual, requiring a skilled welder to control every aspect of the process. This is no small feat as there are a significant number of variables requiring simultaneous action. As noted above, a welder will typically require a helmet with protective lens as the arc that is developed is so bright that extended direct viewing would cause blindness. In addition, the lens is often so dark as to block all ambient light prior to arc strike. Hence, the welder is unable to see the torch or the part before welding commences, making set-up extremely difficult. To alleviate this problem, most helmets either tilt or have lenses that tilt so the welder is able to get into position and then lower the shield. Newer electronic helmets have become available in the last 10 years, however their cost is still substantially higher than considered by most to be practical. These helmets offer the advantage of viewing through a clear lens until the instant of arc strike with automatic dimming in milliseconds to protect the welder's eyes. Traditionally, GTA/TIG welding required electrode contact with the part to initiate the arc, often termed "scratch starting." This process was difficult, as contact longer than an instant resulted in fusing of the electrode to the part with resulting contamination of both the electrode and the stainless material. Newer high frequency "arc starters" function in a fashion similar to lightning by ionizing the local gas allowing the arc to "jump" from the torch to the workpiece without direct contact, provided they are in reasonable proximity based on the voltage potential. During the welding process the arc gap must be maintained relatively constant as the welder traverses the seam to be welded. Virtually any size or shape part must be accommodated, requiring the welder to move longitudinally at a constant speed while maintaining a constant arc gap. Hence, for our purposes, it should be sufficient to note that high quality welding requires, at a minimum; excellent eye-hand coordination, superior reactions, significant stamina, adequate training, and continued practice.

The standardization by pharma on 300 series stainless reminds us of the issues relating to sensitization that can occur. When austenitic stainless remains in the temperature range of 400–800°C for more than 2–3 min, sensitization occurs, resulting in carbide precipitation and significantly reduced corrosion resistance of the material. There are methods that can be employed to minimize the potential for sensitization including rapid welding, with minimum heat build-up, which allows the material to cool quickly enough so that time spent in the sensitization range is below that where carbide precipitation would occur. In addition, low carbon grades of stainless, designated with the suffix "L" can be employed, that by their nature alone, minimize the potential for carbide precipitates, and when applied in conjunction with techniques such as precision welding, multiply the benefit.

A third method is the implementation of automated welding to eliminate the variability created by the manual welding process and ensure, via precise control of heating, the time spent at the sensitization temperature is below that required for carbide precipitation to occur.

Over the decades, numerous methods for automating various welding processes have been implemented. These have ranged from simple fixtured torches to welding lathes, to today's laser guided computer controlled robots. In a factory environment and based on production scale quantities, many component and equipment manufacturers have invested in automated machinery that can improve quality, precision, and yield. Whether creating seam welded tubing, process vessels, machine components, fittings, or filter housings, automated welding is applied to ensure the products produced meet the highest quality standards and are done so reliably. One particular variation of automatic welding, originally developed for the aerospace industry, is termed orbital welding and is

used in the welding of circular materials, most typically tube, pipe, and components similarly shaped.

Orbital welding power supplies typically utilize electronics to control both the power supplied for welding and the action of the special torch that is required for the process. Controllers can range from simple microprocessors to sophisticated computers; however the overall process is relatively consistent from vendor to vendor and relies on the basics of TIG with improvements over the recognized manual process. Torches for orbital welding have been in existence for more than 40 years and are available in two basic types, open and closed. Open orbital weld torches, or heads, utilize a fairly common TIG torch configuration with electrode holder surrounded by a ceramic cup that is used to distribute the inert gas. The torch is attached to a fixture that clamps onto a circular part and precisely rotates the torch head around the part during the welding process. Weld quality is extremely high; however the outside of the weld is typically oxidized since the gas cup only provides coverage immediately adjacent to the electrode. Torches of this type are suitable for tube and pipe applications, structural parts, and machinery components, but do not offer the highest quality available and are therefore not commonly used for pharmaceutical product contact materials unless subsequent machining, polishing, or rework is involved. Alternatively, the enclosed weld torch offers a far superior level of quality based on its unique design. Closed weld heads surround the weld joint a full 360° with inert gas, minimizing any exterior discoloration and oxidation. Some designs even assist by using the exterior purge gas to provide support for the molten puddle, minimizing possible sag that might result in irregularity of the interior surface. In addition to the inherent advantages of these more sophisticated torches, a technique for precise temperature control is commonly employed, termed pulsed arc, whereby the power source pulses the arc at predetermined increments from a high current to a lower, or background current. These pulsations occur in rapid succession allowing the molten metal to cool on each low pulse, effectively creating many overlapping spot welds. The pattern that is created, when graphed, resembles a square wave, and cycle times of 1/10 sec are fairly common. For thicker materials this process can be augmented by a stepping function, called step pulsing that stops the electrode rotation during high pulse allowing greater penetration. Alternatively, for very light gauge materials, speed ramping can accentuate the pulsed arc process by increasing the rotational speed as the material is heated so as to avoid overheating or damage to the part.

Every type of orbital weld head rotates the electrode around the circumference of the part maintaining a precise arc gap, while also offering excellent rotational speed accuracy for superb control of heating. The heads themselves do not rotate and are generally small and compact in size allowing welds to be made in extremely restricted spaces and in virtually any position. Weld heads are designed such that each head can accommodate a range of sizes, minimizing the cost of automation and the amount of hardware required. Control for the torch comes from programming within the power supply, typically downloaded into the machine's memory by the factory or input by the operator. Programming variations range from simple hard wired thumb-wheels, to factory "burned" EPROMS (erasable, programmable, read-only memory) to simple electronic programmers, to computer interfaces, to flash memory cards or memory sticks. Programming allows for the weld to be divided into segments, typically by degrees, with precise control of current, voltage, rotational speed, and time in each segment. Power supplies are often equipped with printers that can provide data that may be useful in validating the quality of the work and may also include feedback electronics to alarm in the event of a problem. Programming over-ride by operators is typically limited to minimize the potential for error and to ensure consistency.

Open weld heads have the ability, as with manual TIG, to fuse two parts together with or without the use of filler metal, which if used, must of course be proper. However, closed weld heads are autogenous in that only butt fusion occurs since the use of filler material is not an option due to their nature. The joint configuration is commonly termed square-butt based on AWS standard terminology and requires precise end preparation so as to avoid gaps that would thin the wall or cause blow-through when welding with an autogenous process. In addition, burrs that would change the arc gap, improperly created tack welds, tungsten inclusions, surface contaminants, and beveled edges can negatively impact the finished weld quality ultimately resulting in rejection.

Automated welding has served to reduce the likelihood of sensitization significantly and, when used in conjunction with "L," or low carbon, grades of stainless, such as 316LSS, has almost completely eliminated carbide precipitation as a concern for most light gauge applications commonly found in healthcare product manufacturing facilities. In spite of the small amount of carbon in stainless steel, its ability to interfere with the corrosion resistance of the alloy is substantial and warrants repeated mention. Worthy of note as well is that other minor constituents can also significantly impact the function of stainless steel. Sulfur for one, at levels below those specified, impedes the welding process by changing the material's heat transfer characteristics, ultimately resulting in widened weld beads that do not penetrate through the metal thickness, potentially causing rejectable weld defects associated with incomplete fusion. As already mentioned, autogenous orbital welding requires very high dimensional tolerances since any small gap between the parts can result in failure, thinning that causes structural weakening, and/or depressions in which organisms may proliferate. To overcome this and other potential problems, specialized tools have been designed to support orbital welding units including cutting and facing tools that prepare the ends to be welded so that they are square and true. Other tools, such as oxygen analyzers, to ensure elimination of oxygen from the weld zone, and borescopes, for internal inspection, are needed to ensure the quality of the finished weld. It is also worth noting that virtually every aspect of the welding process is precisely controlled from verification of materials via Material Test Reports (MTRs), to confirmation of test welds, to the cleanliness of wipes used in preparation, to purity of gas that shields the weld. This is especially necessary for pharmaceutical ingredient or product distribution systems since these welds cannot be polished after they are made and hence must be reliably of the highest quality.

Although discussion of support tools cannot be addressed in full detail within these confines, two specific types justify further comment, including cutting/facing tools and inspection equipment. Tools used to cut or face materials in preparation for welding must typically accomplish two goals; the first of which is creating an edge or surface suitable for the process. This might involve straight cuts, bevels, tapers, or other suitable configuration. Some methods lack the required accuracy or may, by their function, create an undesirable condition. For example, flame cutting typically results in a very irregular surface unsuitable for welding unless additional finishing is performed. Another example is common roller type tube cutters that deform the edge of the material making alignment for welding difficult. The second goal is to avoid contamination or damage to the material. Carbide saws heat the material excessively while creating a substantial burr and often imbed particulate in the surface with a final result particularly difficult to overcome. Cutting and facing operations that operate at relatively low temperatures and without imparting contamination are most suitable. Some of these are able to cut only while others are used for final facing and yet others can do both in a single step. Shop or factory based activity can employ lathes and other

machine tools, while field work can be more difficult especially when repairs in congested or difficult to access areas are the objective. Properly designed equipment is extremely important to ensuring a high quality joint can be completed. Inspection tools are needed when direct viewing of the weld is not possible, and devices range from dental mirrors to expensive and fragile electronic machinery. In any event, care must be taken so that any inspection device that is used does not damage the finish on the material's surface. This is especially of concern when a device must be inserted into a part that has been polished previously and where repolishing is not possible. Early borescopic equipment utilized mirrors and required insertion of a small high powered light into the part, with viewing via an eyepiece much like a telescope. Their design required rigidity, they were unable to navigate corners or bends, and bulbs often broke during contact with surfaces potentially leaving glass shards behind. Advances in fiber optics allowed for remote light transmission, keeping bulbs external to the inspected area, and allowed for flexibility of movement and review of remote previously inaccessible locales. The most advanced equipment in use today employs miniaturized video cameras for high quality viewing on an electronic display as well as recording with data entry capability.

FINISHING

Stainless steels after manufacture, exhibit a dull grey matte finish that may be suitable as-is for use in chemical plants, power generation, and other applications where corrosion resistance is the sole reason for their selection. However, in addition to their chemical resistance qualities, stainless steels when polished, provide an extremely pleasant cosmetic appearance as well as offering a surface that can be easily cleaned for hygienic purposes including that required for food preparation, medical care, and pharmaceutical production. This is not true for all specialized or corrosion resistant alloys, as Hastelloy C-22, often used to replace 316LSS in extremely corrosive environments, is extremely difficult to polish to required levels because of its tendency to foul the polishing tools. Finishing of stainless steel for esthetic appeal can be accomplished using various methods including blasting or peening (as might result from impaction by sand or beads),

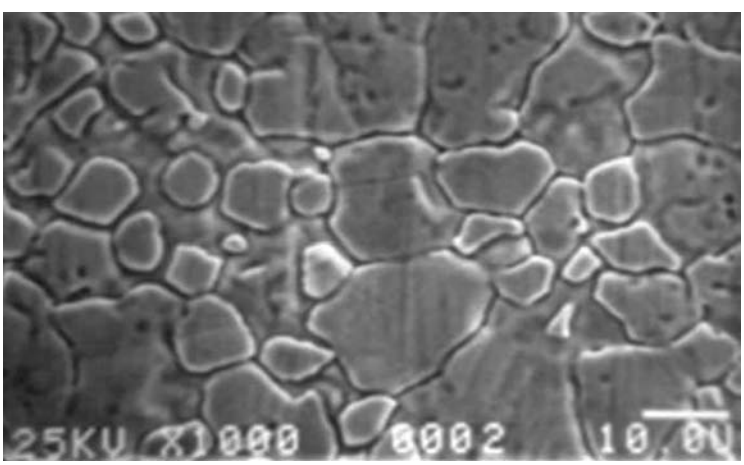


FIGURE 2 Photomicrograph of mill finished stainless steel sheet magnified 1000×. Note 10 μm reference at bottom right.

mechanically using machine tools or abrasives such as emery, or electrochemically using a process known as electro-polishing. It is the latter two that are most appropriate for pharmaceutical product contact and the ones on which we will focus.

The surface finish of materials such as strip, plate, and rod is typically the result of processing, secondary to formulative manufacture, such as forging, hot rolling, or even cold rolling. This finish is commonly referred to as a "mill finish" and although fairly smooth it most likely is granular in make-up similar to that depicted in the photomicrograph, Figure 2.

This quality or level of finish has not been approved in most instances involving food, drugs, or cosmetics primarily because of the crevices that are believed to impede cleanability and sanitation. Through the application of graduated abrasives, applied using various types of belts, pads, disks, wheels, and bobs, the finish can be enhanced so as to be suitable; for architectural requirements including hand rails and door frames, for household appliances and furniture, for marine uses including boat railings and trim, for use in food and drug processing, etc. The requirements for these varied and diverse needs range from basic shininess to precise polishes measured by calibrated instruments to ensure consistent hygienic conditions. To create a high quality mechanical finish, abrasives of decreasing size are progressively applied starting with the coarsest and ending with the finest. Typical finishing for pharmaceutical components will begin with coarse finishing (50–80 grit) with subsequent steps increasing approximately 40–60 grit each until the final required finish is achieved. Grit is used here only to offer the reader a familiar reference since common sand paper is typically specified and labeled by grit size.

Much of the finishing technology used today originated in support of the food and dairy industry where polished stainless steel was utilized for food contact surfaces needing to be hygienic and cleanable. Finishes during that period were typically designated by number such that a dairy finish was considered to be no. 4 or, 150 grit. This meant that the surface, when viewed under a microscope, appeared to have 150 scratches or grit lines across one inch of area, corresponding to the same number of particles of grit on the abrasive tool. This can be seen clearly in the following photomicrograph, Figure 3, which also shows the scouring and smearing of the metal that actually occurs. As is obvious, abrasives used in polishing are aggressive and have a tendency to increase the surface area by creating minute peaks and valleys that increase reflectivity while scoring the surface, much like that of the bygone phonograph record.

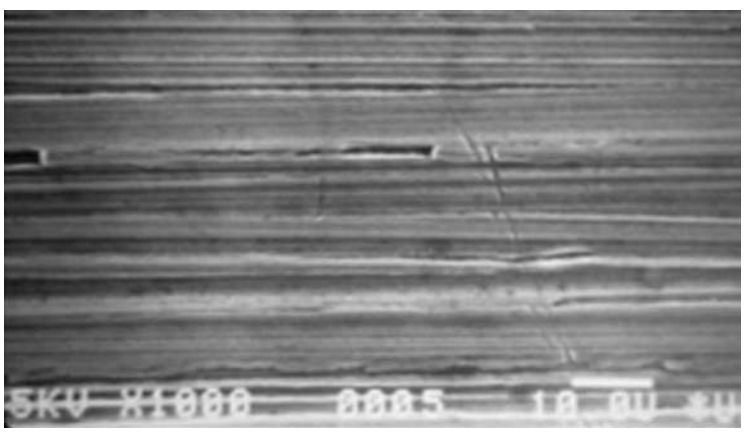


FIGURE 3 Photomicrograph of 180 grit (20 Ra) mechanically polished stainless steel sheet magnified 1000×. Note 10 μm reference at bottom right.

Since finishes cannot be evaluated by the naked eye, products of substandard quality often found their way into the marketplace through unscrupulous vendors trying to gain a competitive edge or buyers hoping for a bargain. Hence, finishes improperly applied as a result of; skipped steps, untrained personnel, or from the use of abrasive tools kept in service beyond their reasonable life, were often indistinguishable from materials with high quality properly applied finishes. One of the significant contributions made by the pharmaceutical industry to sanitary technology was the standardization on roughness average (Ra) surface measurement using the microinch (μm in Europe and elsewhere) scale. This occurred through the joint efforts of vendors and users who desired to create a standard that would ensure equivalent quality and measurable acceptance levels.

In addition, it should be noted that the finishes demanded by pharmaceutical users were higher than that needed by food and dairy facilities, forcing vendors to stock multiple lines of products that were almost indistinguishable, making the need for surface measurement even more critical. As well, measuring tools known as profilometers, that had, in years past, been impractical for other than factory floor use, became available as inexpensive handheld field-friendly devices allowing buyers to confirm the quality of their purchases. Today it is common for buyers to insist that the products they purchase are supplied with documentation verifying the quality of both the material and the finish via MTR's and "finish maps". Materials are coded with "heat numbers" that correspond to the MTR's and finish maps that show the location and value of surface measurements taken at the factory, and can be field verified if necessary or desirable. Today, most materials are physically engraved with much of this information to ensure long term tracking as part of validation is possible. One note of interest is that testing by profilometer imparts a small scratch on the material surface where the test was performed.

Mechanical finishing can be automated, such as for long lengths of tubing, and can also be accomplished by machining as part of a single fabrication process, however not all parts can be automatically polished and items such as the interiors of fabricated tees may require manual finishing. The finesse needed to complete this work requires the operator to not only polish to achieve a smooth finish but to blend with adjacent surfaces to create an appealing final product. More importantly, finishers can easily remove excess material, thinning the wall of the part if too much pressure is applied, or if the tool is allowed to remain in the same position excessively as a result of distraction or inattention. Hence, mechanical finishing remains a combination of art and science as the finished product must meet measurable standards and must look consistent, all while being pleasing to the eye.

Alternative to mechanical polishing is electropolishing, discovered in France in 1929. Electropolishing is an electrochemical metal removal process in which the part to be polished is made the anode and placed into a solution, usually acidic, with a current applied. Metal is removed by dissolution starting from the highest points and with increasing current becomes more aggressive. Electropolishing can also be utilized to electro-machine parts to extremely precise tolerances, as well as for deburring and smoothing in preparation for coating or plating. Common surface reductions of 0.001–0.002 inch during polishing are achieved, however more significant reductions can be accomplished. Electropolishing has also occasionally been termed reverse plating and develops an extremely reflective surface finish resembling a mirror when aggressively applied or when applied over a high quality mechanical finish as is common in most pharmaceutical applications. Electropolished surfaces are distinct in appearance, not at all resembling mechanical finishes, and are extremely smooth with improved cleanability when used with products that adhere to mechanically polished surfaces. Electropolishing offers the added advantage of creating an extremely passive

condition on the metals surface, however there is debate as to whether pre-operational passivation is necessary for materials that have been electropolished. This debate may be appropriate for parts small enough to have been bath electropolished or for assemblies requiring mechanical attachment with minimal chance of damaging the passive surface, however in many instances it is purely an academic discussion relative to a defined surface condition. Let us take for example a liquid product distribution network of 500 linear feet constructed of 1.5 inch diameter sanitary electropolished tube and fittings. This material will require field assembly by welding, however the surface area disturbed by the welding process will be extremely small compared to the entire surface in contact with the product. Discounting fittings to simplify calculations, tubing alone accounts for approximately 179 square feet of surface area that will be in product contact. Based on experience, the number of welds required can be approximated conservatively at 100 with each heat affected zone (HAZ) measuring approximately 0.5 inch in width. Calculation reveals that the area disturbed by welding will amount to 1.49 feet^2 or approximately 0.8% of the entire surface. With less than 1% of the total electropolished surface compromised it may appear that the risks are minimal, however the failure to perform a pre-operational passivation will result in potential compromise of the entire system and not nearly worth the relatively small savings.

In practical field applications pre-operational passivation ideally in-situ is a far safer and practical solution. The following photomicrograph, Figure 4, of an electropolished surface applied over a mechanical finish readily shows the level of increased smoothness that can be achieved.

Smoothness had always been associated with cleanability and assumed to serve as a counteracting force against biofilm formation. There is however much debate regarding this subject. In a recent article published in Medical Device & Diagnostic Industry (MD&DI), Rokicki (2006) states that a new process termed magnetoelectropolishing, which uses a magnetic field to enhance dissolution during the electropolishing process improved the properties of 316L stainless steel and "... found that the magnetoelectropolishing process can add many useful properties, including lubricity and antimicrobial peculiarity to metals used in blood contacting devices. Antimicrobial peculiarity refers to the fact that magnetoelectropolished 316L stainless steel is less prone to bacteria attachment and biofilm formation than standard electropolished 316L steel" (Rokicki, 2006). However, many microbiologists take a contrarians position positing that no surface can successfully resist biofilm formation. Rokicki goes on to state "One big

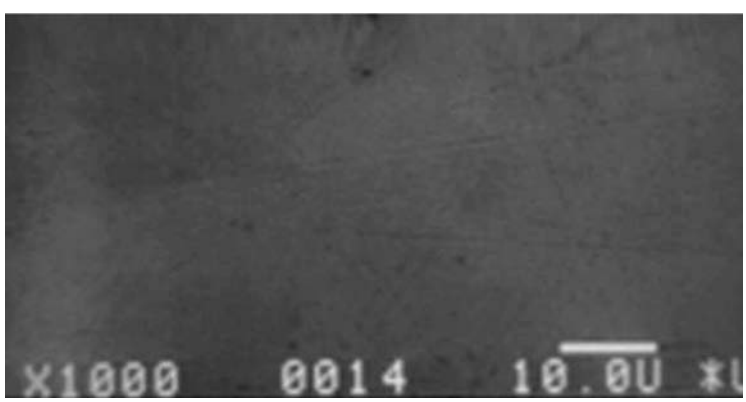


FIGURE 4 Photomicrograph of stainless steel sheet with 180 grit (20 Ra) mechanical polish followed by electropolish, magnified 1000×. Note 10 μm reference at bottom right.

advantage of magnetoelectropolished surfaces of 316L-stainless-steel blood-contacting implants is their improved hydrophilic character. The improvement can be seen in the water contact angle . . . It is well documented that hydrophilic metallic surfaces are hemocompatible and bacteria resistant." As a result of this debate, most likely much research is yet to follow. One very interesting opposing hypothesis concerns the "lotus effect" (Riedewald, 2006) which is based on the surface morphology of the lotus plant. The surface of this particular genus is relatively rough and appears to self clean using water that is unable to adhere to the surface to remove other surface contaminants, thus allowing its surface to remain far cleaner than other plant types. It has been postulated that this geometric phenomenon, if properly applied to a stainless steel surface would render it more likely to resist both contamination and bacterial colonization resulting in biofilm formation. This theory certainly challenges conventional wisdom and is not the first challenge that has occurred regarding this particular subject and most likely it will not be the last.

An in-depth discussion of microbiology would be inappropriate here, however to allow for a complete understanding of the related issues we can simplistically state that organisms occur in either the free-floating planktonic state or preferentially as participants in a colonized biofilm, which affords a more safe and secure environment for propagation. Planktonic organisms reproduce more slowly than their colonized counterparts as a result of their mobile state, are more susceptible to sanitizing materials, and may be more likely to be included when a sample is taken. Conversely, organisms participating in a biofilm have more abundant nutrients, are protected to significant degree from sanitants, and in their stationary state are relatively secure from sampling, entrapment, or other exposure to system hardware. Biofilm occurs ubiquitously in nature and is most readily recognized by anyone attempting to cross a brook by stepping on submerged rocks. The incredibly slippery surface that exists usually results in a fall since firm footing cannot be achieved.

Most biologists believe that biofilm cannot be avoided; however frequent sanitization of one form or another can reduce its rate of development. There has been significant research in this field although pharma specific information does not appear to be as available and this suggests further biofilm related research would be appropriate. Based on available information and historical performance of pharma water systems, this author believes that biofilm formation can be significantly retarded when continuous sanitization is employed such as for systems operating continuously above 165˚F, and that there is a direct correlation between occurrences of sanitization and biofilm formation.

FABRICATION OF STAINLESS STEEL

Welding is the primary method of permanently joining pieces of stainless steel and results in homogeneity that can be subsequently finished to create a smooth cleanable surface if properly created and polished. Alternatively, stainless can be fabricated using numerous other techniques, such as machining, drawing and bending, alone or in concert, and also in conjunction with welding. In fact, it is common for assemblies to be the result of multiple fabrication steps which can include many of those listed above. Take, for example, any standard single element filter housing consisting of tubesheet and head. The tubesheet assembly is the point of connection of the element and usually contains the connection fittings. It is most likely constructed from an appropriately sized piece of plate that is machined, possibly on automated equipment. Connecting the tubesheet to the

process requires fittings and these may be internal ports machined into the tubesheet, usually threaded for non-sanitary applications, or fittings welded and polished appropriately for sanitary applications. In addition, hold-down tabs and other accessories may also be required for the particular application. Alternatively, the tubesheet may be constructed as a stamping from thinner material, or may be completely machined including fittings, from a single cast or forged piece. Either of these options impact the cost with higher prices associated with the machined component and lower associated with the stamping.

Accessory parts, such as hold-down tabs may be machined or formed, as required by the application, for welding to the head and subsequent polishing. Dome or head assemblies can be fabricated from parts or may be formed from a single sheet possibly using a deep drawing operation. Head fabrication from parts can include the body, cap, fittings, valves, mounting fitting/flange, and other specialized items typically welded and polished for sanitary and even non-sanitary units. Where possible, standard subcomponents will reduce the cost of manufacture and hence, improve competitiveness. Housing body material can range from standard diameter tube and pipe, prepolished, to virtually any custom diameter created form sheet that is rolled and welded and can even be fabricated in sections if required. Caps can be closed or open, depending on the need for fittings, and can be formed, machined, or drawn. In some product lines, reducers can serve to simplify manufacturing or for more complex designs, special fabrications may be necessitated that incorporate centering devices or retainers.

For non-sanitary applications, parts could be threaded, bolded, welded, or assembled in any fashion that accomplishes the physical prerequisites including mechanical and hydraulic integrity, serviceability, and operating pressure. For sanitary applications the added requirements of cleanliness dictate the use of sanitary fittings, polished surfaces, drainability, appropriate sealing, and possibly integrity testing.

Vendors may require specialized equipment to facilitate the fabrication of their products which may or may not be commercially available. Special welding fixtures as well as unique tools for polishing may be the only practical solutions to overcoming fabrication difficulties and allowing a product to arrive in the marketplace. Stainless steel will remain our primary focus, however it should be noted that alternative materials are certainly suitable for housing construction, including various other metals as well as a multitude of plastics, all based on process worthiness. For drug product contact however, stainless steel remains the most accepted. The example housing included above is simple and appropriate for discussion of pharmaceutical products, but is hardly representative of the entire range of products available. It would take the reader only moments using the Internet to locate dozens of manufacturers and to view the myriad of designs, styles, sizes, shapes, and applications that exist, however it should be recognized that the vast majority of these are not accepted for use in sanitary environments.

As with most other applications for stainless steel, the primary issues are formability, welding, and polishing. Welding as discussed before is performed primarily by the TIG process, using both manual and automated configurations, although specialty welding including welding lasers, resistance welding, and plasma arc welding are also employed. Advantages can be gained from the use of specialized welding as often increased speed, reduced HAZ, smaller weld bead (for ease of polishing), and minimized warping result in higher quality product and decreased cost.

The physical characteristics of stainless steel promote the desire to weld more quickly and with less heat input so as to minimize distortion that can become problematic if uncontrolled. With its melting point below that of common carbon steels and with lower coefficient of thermal conductance, higher coefficient of thermal

expansion and higher electrical resistance distortion can easily become a fabricators dilemma. Even simple functions, such as welding two tubes together, can result in difficulty if consideration to the nature of stainless is ignored. Many tube welding failures have occurred when untacked or lightly tacked materials pull apart because of shrinkage and distortion. Plate and sheet materials also often distort during fabrication such that they are rendered unusable, especially if the fabricator is unfamiliar with proper techniques. Maintaining dimensional accuracy when fabricating stainless, is a unique challenge that can be overcome only by experience, knowledge, and proper equipment.

In addition, it is not uncommon for stainless welding to be improved by a stepwise approach rather than creation of a single bead. When welding stainless steel, regardless of the thickness, it is prudent to consider this approach to avoid warpage that might otherwise occur. Many vendors of parts recommend a crisscross approach that requires (using a clock face for explanation) division of the weld into a number of segments that distribute the heat to avoid damage to or warpage of the part. For example, welding might begin with approximately 1/12 of the weld created at 3 o'clock, and subsequent equal sized sections welded sequentially at 9, 6, 12, 4, 10, 7, 1, 5, 11, 8, and 2 until the seam is completed. This process has proven extremely useful in welding large pieces of plate, small light gauge parts, or components of differing thicknesses, although the exact pattern of creating the joint may vary from part to part and fabrication to fabrication.

Stainless exhibits excellent mechanical workability such that it can be drawn, bent, and machined with relative ease. Hence, it is common to see stainless components ranging from ductwork, to punches and dies, to step ladders, all more expensive on a first-cost basis but with far superior overall life expectancy. For this reason, stainless can form the tubesheet, head, fittings, clamp, and even the accessories for a common filter housing with little fanfare.

In addition, the physical properties of stainless also allows finishing, either mechanically or electro-chemically, to a superior quality and offers brightness when finished, substantially above most other materials, including platinum, with the notable exception of silver. Finishes applied to stainless are done so in a cost effective manner providing benefits that can offset the better corrosion resistance of more exotic alloys such as hastelloy. It is the corrosion resistance, ease of fabrication, relatively low cost, and availability of stainless steel that makes it such an attractive overall alloy, with few competitors offering a challenge to its position as material of choice for pharmaceutical, biotech, food, beverage, cosmetic, and dairy applications.

CONCLUSIONS

When compared to other metals, especially steel and iron, the entire family of stainless steels accounts for a very small portion of the world's overall metal usage, so small in fact that stainless steel accounts for significantly less than 5% of the overall volume. This should not be surprising as the largest applications for metals such as steel and iron involve bridges, buildings, transportation (cars, trucks, buses, trains, ships, etc.), machinery, and other applications that consume incredibly huge amounts of material on an annual basis. Stainless steel is unique, multifaceted, and surprisingly inexpensive, yet there are few industries that can justify the added cost or overcome the obstacles that range from planned obsolescence to personal preference, although stainless does tend to be viewed as a modernistic material of construction.

It would indeed be an interesting exercise to compare the cost of bridge construction in stainless steel against the life-cycle cost of maintenance including parts replacement due to rusting and repeated paintings with the associated environmental impact. Likewise, machinery, automobiles, trucks, trains, and ships would never corrode and would never require painting if they were made from stainless steel as is the case of the Chrysler building and even the DeLorean automobile, although admittedly, it might get a bit tiresome if everything lasted indefinitely and only came in one color.

In closing this chapter, it is worth mentioning that stainless steel is a remarkably unique material with an extremely long life compared to mild steel. Many stainless components, used by industry and by homeowners, even though repairable or reusable, will outlive their practical usefulness and require replacement. Often this material is discarded into landfills and other inappropriate dumpsites. Stainless steel is 100% recyclable, and as such, its disposal as basic garbage or into landfills is an unforgivable error that should be avoided at all costs.

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10
Protein Adsorption by Polymeric Filters

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INTRODUCTION

The adsorption of proteins onto polymeric filters is of considerable practical interest. The technique of preparing new drugs by way of fermentation processes includes their downstream purification by separation procedures such as filtration or liquid–solid chromatography. Separation of the extracellular products from the cell remnants is achieved by filtration in a clarification step; microporous membranes are employed. The debris is retained by the filter; the effluent contains the desired proteins. Ultrafiltration by way of tangential flow operations is a technique often used to concentrate the sought-after products. Alternatively, liquid-solid chromatography is utilized to bond the proteinaceous products to a carrier surface. Subsequently, the desired (protein) product is released in purer form.

Purifying activities based on filters can be complicated by blockage of the filter pore caused by protein and/or other depositions. The same may result when preparations containing proteins undergo filtrations designed for sterilizations, and for virus removal. The hindrance of the filtration process by protein adsorption is termed "fouling" a word whose pejorative implication is one of unwelcome interference. Actually, fouling is a poorly understood phenomenon. Protein adsorptions studies are undertaken in pursuits of its amelioration. The loss of time and effort attendant upon a filter blockage translates to a monetary loss. An even greater direct loss may occur when valuable proteins, the product of present biochemical efforts, are irreversibly adsorbed to a filter to become discarded with it. The strong interest in securing high yields of high-value proteins is self apparent.

The technical literature is not as clear as it might be regarding the type(s) of filters most responsive to protein bonding. Commercial competition for the filter market may, on occasion, tend to blur differences among filters regarding their adsorptive properties. One of the aims of this chapter is to provide technical guidance that may be of value in reaching independent judgments.

The factors that govern protein adsorptions to filters are broadly understood. A review of the mechanisms involved will be made with an eye towards matching them with salient features of the filters' polymeric structures. A clearer understanding of the adsorptive bonding of proteins to filters is its goal.

PROTEINS AND ADSORPTIONS

Analysis of data derived from experimental studies of protein uptake by membranes indicates that sieve retention, especially of aggregated or denatured protein, is an

operational mechanism (Sundaram, 1998, pg. 548; Kelly et al., 1993). By far, however, protein uptake by filters is an adsorption phenomenon (Zydney, 1996).

The adsorption of proteins can occur whenever any surface comes in contact with a protein solution. Protein adsorption to filters is hardly a singular or exceptional event. Various particle types such as organisms, colloidal particles, humic acids, and numerous molecular substances of organic-chemical origins, preservatives, stabilizers, endotoxins, etc., undergo similar bonding. Nor do filters supply the only surfaces for adsorptions. In fact, large surfaces, whether of activated carbon, or beds of ion-exchange resins, or sand, qualify equally. It is the extent of surface, among other factors, that governs the quantities of materials adsorbed. However, the particular properties of a given molecular structure also exert an influence on how it behaves in the adsorption process. Thus, silica glass made from silica sand, despite its greatly reduced surface area compared with the sand granules, is also available for adsorptive interactions. Therefore, the loss of protein from solutions stored in glass vessels also deserves appraisal.

When an organism adsorbs to a filter, or when colloidal particles agglomerate, the result is seen as a surface-to-surface reaction. A different terminology is needed to describe the adsorption of a molecule to a filter surfaces. Albeit three dimensional, molecules are ordinarily not thought of as having surfaces. Yet the forces that adsorptively unite the two entities are of an identical nature. The interacting molecules may each be part of a surface. They may also be in solution as macrosolutes, certain atomic arrangements of which mutually attract and bond by way of adsorptive mechanisms that will be described below.

It is proteins in the form of solutions that are processed by filtration. Often, however, because of their structural complexity, proteins occur as amorphous or colloidal particles. They are essentially rather complex combinations of amino acids, sometimes including linkages to other type molecules. The amino acids, as the name implies, are each characterized by their terminal groups; one an amine ($–NH_2$), the other a carboxylic acid group (–COOH). As a result of being amphoteric, they combine respectively with both acids and bases. The amine and carboxylic acid groups of separate amino acid molecules can be interacted to form amides. The resulting larger polypeptide molecules may also be characterized by terminal amine and carboxylic acid groups. A cascade of repetitive interactions with other amino acids can yield convoluted long chains of high molecular weights composed of the various linked amino acids and possibly of other substituent groups of atoms. The resulting proteins are repetitive units of their constituting amino acids transformed into polyamide chains, all connected by a series of amide linkages (–C–N–).

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ISOELECTRIC POINT

All large molecular surfaces acquire a net surface charge in aqueous solution due to the adsorption of positive or negative ions from the liquid medium and / or the ionization of chemical groups on their surface (e.g., the ionization of carboxylic acid groups which form when organic molecules are oxidized to yield COO^- anions). As a result their large surfaces, many particles come to exhibit a net electrical charge. At neutral pH, most membranes preferentially adsorb negative ions (Zeman, 1996: Chap. 4)\ to acquire a net negative charge. This negative charge increases in alkaline solution, above pH 7, whether

from an increase in anion adsorption; an increase in the ionization of acidic groups such as the carboxylic acids, or from the deprotonation of basic groups, such as amines (e.g., $-NH_3^+ \rightarrow -NH_2$).

The reverse behavior is true in acidic solution. As the solution pH is lowered below pH 7 increasing quantities of plus charges are adsorbed or generated by way of ionic dissociations that yield hydrogen ions. These through hydrogen bonding[a], (explained in the following section), join with water molecules to form hydronium ions, (H^+ - - - H_2 O) or (H_3^+O). most amphoteric species will eventually pass through a point at which they have no net charge. The transition from the negative to positive state identifies the pH where there are essentially no charged molecules. The pH at this point is referred to as the isoelectric point or pI. It differs from the neutral point because of the effects of the adsorbed or generated charges just discussed.

The zero potential is practically null, and there is, therefore, little if any trendency for the molecule to migrate in an electric field. Free of electric charge influences, the iusoelectric point identifies the pH at which the particular protein is to a maximum degree responsive to adsorptive influences.

The isoelectric points of proteins range from a low of pI < 1 for pepsin to a high of pI=11 for, lysozyme. This wide range of pI reflects the impressive heterogeneity of armino acid compositions among different proteins (Zydney 1996). The heightened interactions of filters with proteins at the charge-free isoelectric points of the latter are taken to indicate hydrophobic adsorptions.

Given the importance of proteins, it is perhaps not surprising that they have undergone considerable investigation, both experimental and theoretical. Consequently, there exists a body of knowledge concerning how these entities undergo interactions resulting in their adsorption to one another, and to surfaces such as they encounter in filtrations. Protein adsorption is a multi-faceted process. It is dependent upon the individual protein, upon other proteins that may be present, the protein concentrations, the makeup of the specific liquid vehicle such as its ionic strength, pH, etc., and upon the process conditions, such as buffer composition, temperature, etc.

CONFORMATIONAL CHANGES OF ADSORBED PROTEINS

In being adsorbed, the protein's molecular structure tends to conform to the shapes of the adsorbing surfaces. In so doing, its shape and certain of its properties are altered. This may include a reduction in the extent of biological activity. The degree of conformation presumably is a measure of the strength of the adsorptive bonding. There is a direct commonality between a protein's hydrophobicity and its adsorptive conformation to the substrate. In attaching to a site on the filter surface the pliable protein molecule may fold on itself. In so doing it exposes small portions of its more hydrophobic amino acids. These are shielded when the protein is in aqueous surroundings. The resulting increase in its hydrophobicity may promote additional folding and shape-alteration as the protein molecule interacts with more adsorption sites.

In addition to the initial steric factor, the hydrophobicity of a protein progressively increases in several successive stages of folding. Its primary structure reflects its molecular composition. Its secondary, tertiary, and quaternary stages represent its three-dimensional structures wherein the hydrophobicity increases as also its conformation. Under strong

[a]The hydrogen bond is often represented by three dashes (- - -).

adsorptive interactions at hydrophobic sites, given time, protein molecules tend to flatten into a very dense layer to an extent shorter in length than that of the molecule in solution (Sundaram, 1998). They increasingly undergo more avid surface interactions as expressed by greater extents of conformation. Conformational changes are favored by an increase in a protein's flexibility, as shown by adiabatic compressibility studies.

The protein deposit once formed is not necessarily reversible. Under conditions that favor strong adsorption, desorptions may prove irreversible. Such reversibility is more likely where the adsorption is of a hydrophilic type, presumably because such are less tightly held. The adsorption can be reversed by changing the solution conditions to those that are promotive of hydrophilicity. This can be effected by the presence or additions of alcohols, glycols, and surfactants to the aqueous solution. Such will change the character of the adsorption from hydrophobic to hydrophilic. Desorption is then more possible. Following desorption, the protein may recover its original conformation either partly or completely. The greater strength of the hydrophobic adsorptions is indicative of the greater stability of the protein in those very conditions.

Truskey et al. (1987) measured protein adsorption, circular dichroism (CD)[b], and the biological activity of a variety of protein solution, namely, of insulin iso-gamma globulin (IgG), and alkaline phosphatase. The solutions' properties were measured before and after passing the proteins through a variety of membranes. Shifts in CD and decreases in the activity of the enzymes were determined to be the result of conformational changes of the protein structure that are attendant upon adsorption. This study showed that membranes with the greatest degree of hydrophobicity had the greatest effect on protein adsorption. This, in turn, effectuated a strong conformation with concomitant denaturation. The protein–membrane interaction resulted in the protein's exposing its internal hydrophobic sites, which were folded within its structure during its exposure to aqueous solution. This bespeaks a relationship between protein shape and function. The exposed hydrophobic sites that engage in the membrane–protein binding, protein–protein binding, and protein denaturation are likely the more hydrophobic amino acids; the most hydrophobic of which are tyrosine, phenylalanine, and tryptophane.

Opportunities exist for the shearing of protein molecules as they negotiate a microporous membrane's tortuous passageways. A loss of the protein's properties may result. (Such, at least, are the fears of wine aficionados who decry wine filtration for the taste changes they allege to occur, presumably as a result of protein shearing.) Shearing in general is, however, seen as being less responsible for functional losses through denaturations than are the conformational changes that follow the adsorptions of proteins to polymer surfaces.

ADSORPTION ISOTHERMS AND FOULING

According to accepted theory, the atoms composing the surface of a solid are arranged in the orderly fashion of a crystalline lattice in a checkerboard pattern. Each atom

[b] In circular dichroism, differences, if any, in the adsorption by protein of right and left polarized light are measured before and after filtration. Differences in the CD spectra bespeak structural alterations resulting from the protein molecule's conforming to the shape of the surface upon which it adsorbs. Such conformation can result from protein-ligand interactions, and from the effects of denaturants such as pH, temperature, surfactants, or shearing. The spatial asymmetry in the amino acid distribution is manifested in the secondary, tertiary, and quaternary structures of the protein.

comprising the surface has available free valence electrons capable of interacting with one, and only one, molecule from its surrounding phase, be it a gas or a solution. The rate of molecular adsorption depends upon the rate at which molecules collide with the surface, the fraction that adhere, and the extent of surface area that becomes covered. The relationship of these several factors is known as the Langmuir isotherm. In its accord with the data from many adsorption studies, it is considered the archetype of the classical adsorption. The data forthcoming from the studies of protein adsorptions onto filter surfaces would have been expected to fit the Langmuir isotherm equation. One of its assumptions holds that only the one layer of protein molecules directly in contact with a filter's surface is adsorbed upon it. This is not found to always be the situation.

Experimental investigations indicate that elements of protein adsorptions to filters do fit the Langmuir equation. This argues in favor of its underlying assumption, namely, than only one layer of molecules can be adsorbed. However, so thin a deposition ought not be enough to lead to membrane fouling. Also experienced are fouling situations wherein filters speedily suffer flux decay consequent to adsorbing only minute quantities of aggregated or insolublized, or denatured protein. The more orthodox view of fouling is the progressive building of an adsorbed layer to the point of pore-blockage over a longer term filtration. Such an occurrence is not expected in Langmuir type one-layer adsorptions. As will later be discussed, Bowen and Gan (1991) made use of the standard Langmuir model in analyzing their adsorption isotherms. It did not provide a reasonable fit for their data. The present situation is far from clear.

Adsorptive Forces

According to Zeman (1995, Chap. 4) and Zydney (1996, Chap. 9), the interactions between filter and adsorbent arise from a number of causes. Electrostatic influences involve the molecule that is adsorbed sharing the partial-electric charge of one of its atom-sites with a partially-charged atom of a molecule of opposite sign on the filter's surface. The partial-charges are generated by fixed charges, as of ions, or by fixed dipoles; also to be explained. It is this interaction that marks the adsorptive bond. Adsorptions may also result from forces that, according to some authorities, are not necessarily charge-related. These are known as hydrophobic adsorptions. Their motivation is ascribed to reductions in Gibbs free surface energy.

There are also polarization, or London–van der Waals (VDW), forces that derive from induced dipole–induced dipole reactions. The nature of the covalent bond will presently be described. Conformational changes are favored by an increase in a protein's flexibility, as shown by adiabatic compressibility studies.

Heterogeneity of Surfaces

The bonding interactions that characterize adsorptions are understood to be electrical in nature. (There is some disagreement regarding the hydrophobic adsorption mechanism.) Electrical charge considerations, partial-charges, hydrogen bonding, dipoles, VDW forces are all involved. The electrical potentials that result may empower given atoms with positive or negative charge characteristics. The resulting opposite or like-charges are essential parts of the adsorption phenomenon. Adsorptions to filters can be considered as interactions between their surface molecules and certain features of the molecules present in their surrounding environment. The chemistry of proteins and of

surfaces to which they adsorb are often heterogeneously complex to an extreme. Various sites on a surface may be partially-charged and can be of like- or of opposite signs. The extent of charge-site hydration, for example, is influenced accordingly. Their electric potential, and the hydrophobic/hydrophilic character of given atomic sites on any of two surfaces may differ considerably. Importantly, their tendency for adsorption may also manifest itself to different extents.

Protein adsorption is a sum total of many phenomena. As a result, it is difficult to predict. Protein surfaces can contain differences in hydrophobicity, charge, and degree of hydration, and can change with protein conformation, and solution characteristics (Zydney, 1996). The filter surface to which it bonds in its adsorptive interaction may have similar differences. Neither surface need be uniform with regard to surface charge or surface composition. Opportunities simultaneously exist, therefore, for both hydrophilic and hydrophobic adsorptions to take place. The former involve electric charge interactions; the latter presumably are free of such influences. Both may simultaneously occur in a given situation. It is possible to ascribe a given adsorption to either one of these causes, or to both.

Hydrophobic versus Hydrophilic Bonding

It is possible to make an assessment regarding the relative strengths of the charge-involved hydrophilic adsorptions and those of the hydrophobic interactions that are, presumably, charge-free. A number of situations suggest that the hydrophobic adsorptions are the more effective:

1. The adsorptive interaction of a protein and a surface increases as the hydrophobicity of both filter surface and protein increase. Moreover, as electrical charges on the protein and on the adsorbing surface decrease, adsorption generally increases. Therefore, protein-filter interactions are generally believed not to depend upon electrostatic attractions. They are credited to hydrophobic forces. (Hydrophobic adsorption is considered by many authorities to operate free of such electric charge influences.)
2. Desorption is a function of the rate of evaporation from a completely covered surface. As stated above, the reversibility of an adsorption is more likely where it is of a hydrophilic type, presumably because such are less tightly held. Desorption may result from changing the solution conditions to those promotive of hydrophilicity; as by additions of alcohols, glycols, and surfactants to the aqueous solution. Desorption from the hydrophilic bonding is then more possible. The greater strength of the hydrophobic adsorptions is indicative of the greater stability of the protein in those very conditions.
3. It is known that adsorptions are greatest when proteins are at their pI points, at which pHs they are essentially free of the ionic charges gained them by their amphoteric amine and carboxylic acid groups.

An exception arises when the dominant ionic groups on the protein and filter surfaces are of opposite electrical signs. In this case, an increase in protein adsorption follows directly from a decrease in the repulsive aspect of the zeta potential. The attractive forces are then enabled to exert their influences. This situation results from the "screening" of the repulsive charge by high ionic strengths such as eventuate from salt additions, or from the higher hydronium ions of lower pHs.

Protein Shape Alterations

The picture of the absorption process is not one of particles or protein molecules encountering and becoming bonded by chance to a surface in some static fixation. Molecular movement is involved. The attractive forces making for adsorption, both VDW and hydrophobic, are effective only at very short ranges. Where the distance between the two surfaces is small enough for bonding to occur, the protein molecule will attach end-on. Conformational changes, movement within the protein molecules, will then result. The protein molecule will tend to collapse by undergoing progressive stages of folding, and to flatten out into dense layer on the membrane or other solid surface. In the process its hydrophilic/hydrophobic character may alter as described above.

Charge attractions may not become manifest because the distance separating them are too great. Even so, they can become operative in high ionic strength situations. This is so because high ionic values, in effect, condense the electrical. As stated above, shrinking them to distances wherein charge or hydrophobic attractions can assert themselves. Movements, both inter- and intramolecular, are part of the adsorption picture. In this type of dynamic setting there are possibilities for adsorptive bondings to take place.

It is intriguing that the outermost atoms (surfaces) of a complex molecular structure presented by a complex protein molecule may undergo significant property changes depending upon the degree to which its convoluted molecule becomes extended or contracted in solution. Protein molecules display their most hydrophilic character in their extended, if sinuous, forms in aqueous solutions. Their solubility in water derives from the prominence of their hydrophilic amino acids in aqueous solutions. In the situation, their constituting hydrophobic amino acids are least exposed to the water. As their structures fold progressively to where their more hydrophobic amino acids become more openly exposed, they undergo an increasing hydrophobicity. In consequence, this tends to encourage hydrophobic adsorptions; soon to be discussed.

As discussed below in the section entitled Fuoss Effect this very alteration of molecular structure mirrors the furtherance of humic acid adsorptions onto activated carbon surfaces by the presence of ions. This exemplifies the wide application of the adsorption process that also governs the protein-filter picture.

Another example of the general nature of the adsorptive interaction was related by Brose and Waibel (1996), while discussing their work on protein adsorptions by filters. That preservatives can be adsorbed by filters is known. These investigators reference their findings with regard to the adsorption of certain preservatives, namely, benzalkonium chloride and benzododecinium chloride to filters. The subject adsorptions and concentrations reflected linear relationship at the 0.02–0.25 wt % level.

Hydrophobic Adsorption: Serum

A number of protein-to-filter adsorptions will be discussed with a view towards identifying the operational factors, such as were just described.

The ease of filtering serum depends largely upon the animal species from which the serum is obtained. Several of the many components of sera add to the difficulty of their filtration. Fetal calf serum is relatively easy, and porcine serum is relatively difficult to filter. The most troublesome components are the lipids, and proteins. When their depositions and adsorptions block and clog the filters, the occurrence is called “fouling.” The contribution made to filter blockage by the membrane’s polymeric composition is pronounced when bovine serum is the filtration fluid. Comparison was made between a 0.22-μm-rated cellulose triacetate membrane and a 0.45-μm-rated experimental PVC membrane as a filter for bovine serum. In order to minimize clogging due to particulate

matter, the bovine serum was prefiltered through a 0.8-μm-rated cellulose triacetate membrane. It was found that the cellulose triacetate membrane, although characterized by a smaller mean-pore size, yielded double the throughput of the experimental PVC filter. The difference in performance is ascribed to filter interaction with the serum proteins. Protein binding "fouls" the hydrophobic (PVC) membrane and reduces the throughput.

Such proteins as the serum albumins undergo hydrophobic interactions with the long non-polar chains of fatty acids that terminate in hydrophilic carboxylic acid groups. In the case of the interactions between the fatty acids and serum proteins, the opportunity for hydrophobic interaction is increased by muting the polarity of the protein. This is done by adjusting the pH to the isoelectric point of the particular protein, because at that point the electrostatic charges inherent in the amphoteric protein molecules are neutralized. That the elimination of partial-charge effects does not vitiate protein adsorption based on hydrophilic surfaces serves as support for the hydrophobic adsorption mechanism.

Hydrophobic Adsorption: Ultrafilters

Ultrafilters of various molecular weight cut-offs (MWCO) were tested for their abilities to remove endotoxins. The membranes were composed of polysulfone, and of cellulose triacetate (Wolber et al., 1998). Log reductions of 4–5 were obtained using filters having MWCOs of 10,000. The endotoxin removal resulted from hydrophobic adsorption rather than from sieve retention.

All known endotoxins have a hydrophobic lipid A core as well as a hydrophilic polysaccharide appendage. It is the lipid A core that is responsible for the pyrogenic activity of the endotoxin and for its hydrophobic character (Galanos et al., 1972). Its non-polar nature reflects the 16–20 carbon chain that it contains. This non-polar chain furnishes the site for the hydrophobic interaction between the lipopolysaccharide and the like-bonded segments of filter surfaces, the non-polar polypropylene molecules.

Lipid A, being non-polar, is incompatible in terms of phase separation with aqueous solutions. It spontaneously aggregates in such media to form small micelles or larger bilayered vesicle arrangements (Sweadner et al., 1977). In these formations the negatively charged hydrophilic groups lie on the surfaces of the molecule exposed to the aqueous solutions. The core of the micelle or vesicle is composed of the hydrophobic lipid-A portion of the endotoxin. The exposed anionic groups on the outside attract their oppositely charged counterions to form an electrically neutral double layer that also includes water molecules bound by dipole–dipole interactions.

Robinson et al. (1982) (Fig. 1) illustrates the lipopolysaccharides (LPS; endotoxins) pyrogenic material being adsorptively retained by hydrophobic membranes through the hydrophobic interaction of the uncharged filter surface and the non-polar lipid-A core of the endotoxin. The enclosed non-polar lipid-A core establishes contact with the non-polar polypropylene pore walls and so becomes hydrophobically adsorbed upon rupture of the charged hydrophilic pellicle. At the bottom of the hydrophobic effect is the entropically driven tendency of hydrophobic structures to interact with one another in order to reduce the area of their contact with water. This is attended by the free surface energy reductions which some consider the prime cause of hydrophobic adsorptions.

Hydrophobic Adsorption and/or Sieve Retention

The endotoxin molecule has a distinct chemical characteristic in that the O-antigen at one of its ends is hydrophilic, while at its other end the lipid A portion is very hydrophobic (Fig. 2). This dual nature allows the pyrogens to aggregate into micelles

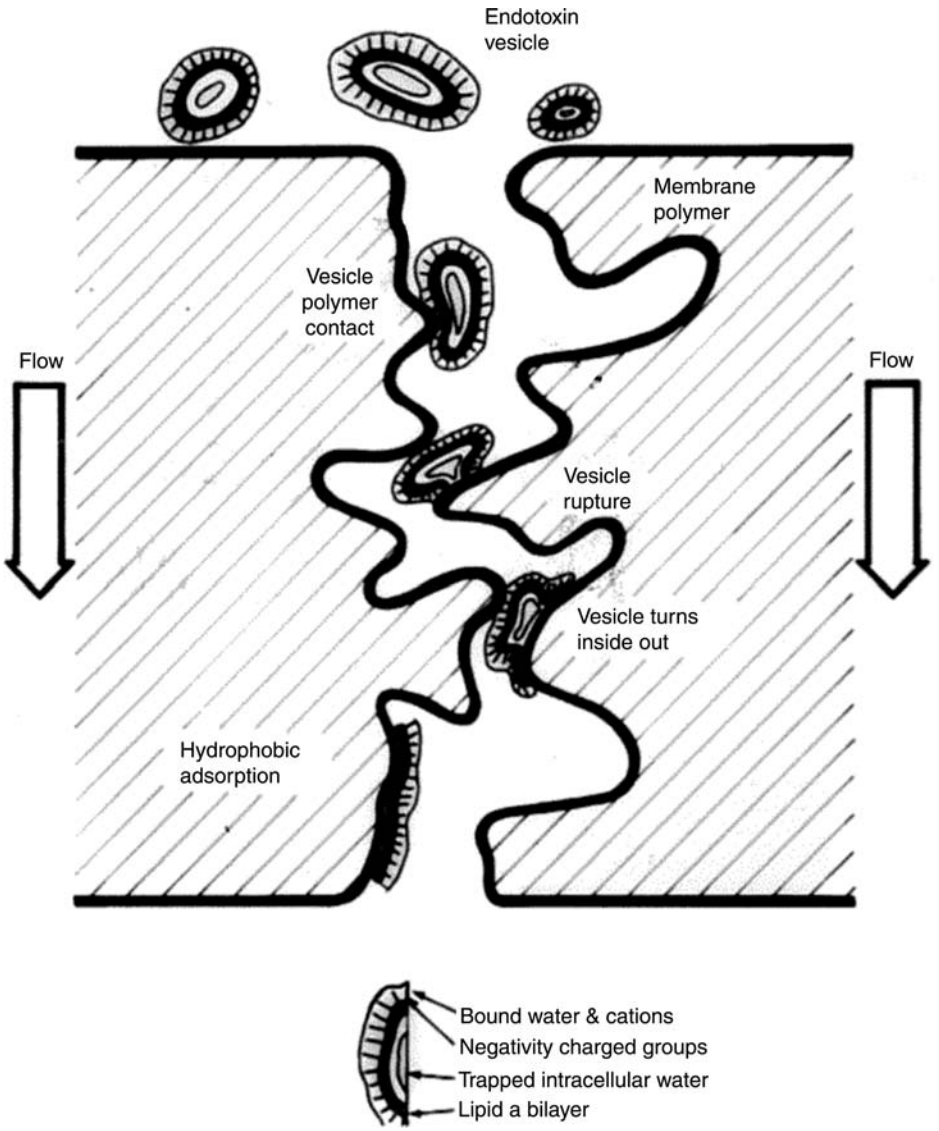


FIGURE 1 Hydrophobic adsorption mechanism. *Source*: From Robinson, 1982.

and vesicles, depending upon the composition of the solution (Table 1). If divalent magnesium or calcium ions are present, the aggregation of micellar units forms vesicles large enough to be submicrometer in diameter. If the divalent elements are removed by chelating agents such as ethylenediamine tetraaceticacid, the micelles will revert to molecular weights of 300,000 to 1,000,000 Da. The action of detergents on the micelles results in their division into individual LRS subunits of 10,000 to 20,000 Da (Sweadner et al., 1977). Presumably, sieve retention of any of the LPS units would result from the use of filters of suitable pore sizes. However, the state of LPS aggregation is not always known. This need not be of practical concern. The strong tendency of the LPS to adsorb to surfaces can account for their filtrative removal by hydrophobic

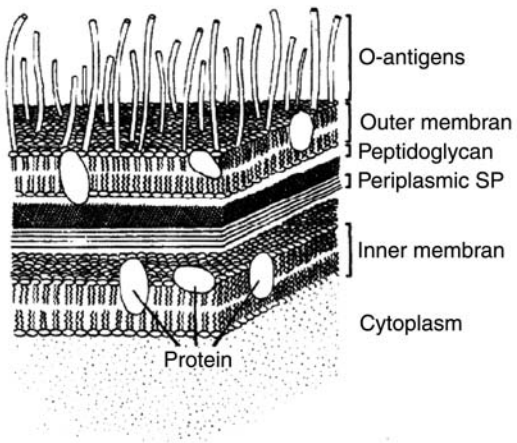


FIGURE 2 Outer coats of a Gram-negative bacterium. *Source*: From Gabler, 1978.

adsorption; sieving aside. LPS adsorptions to surfaces are, to some degree, inevitably irreversible. Water and saline solution generally facilitate adsorption.

Table 1 illustrates that the removal of the endotoxin, as measured by log reduction value (LRV), can indeed be inversely proportional to the pore size of the filter. Apparently, the sieve retention of endotoxin can take place when filters of a proper small pore size are used. Being polar, the *hydrophilic* cellulose acetate (CA) filter is presumed not to participate in hydrophobic adsorptions. Gould (1998) opines that "positively charged filters often are more efficient at adsorbing negatively charged endotoxins". Be that as it may, positively charged filters, described in an earlier chapter (Charge-Modified Filtration Media) operate on a stoichiometric basis. Each positive charge site can remove one negatively charged endotoxin.

According to Gould (1998), "These filters 'work' until the surfaces are saturated with endotoxin; therefore, their use requires periodic monitoring to ensure that endotoxins do not break through." The eventual saturation of the positive charge sites

TABLE 1 Influence of Pore Size and Endotoxin Aggregation State on Endotoxin Adsorption to Filters

Polymer type	Pore size (μm)	Endotoxin aggregation state	Endotoxin LRV
Polypropylene	Prefilter	DI	0.1
	0.2	DI	1–3
	0.1	DI	3–4
Cellulose acetate	0.2	DI	0.1
	0.025	DI	3.0
Polypropylene	0.2	EDTA	1.0
	0.1	EDTA	1.0
Cellulose acetate	0.2	EDTA	0.1
	0.025	EDTA	0.1

Notes: (1) Deionized water (DI) solutions of endotoxin are mixtures of vesicular and micellar structures. (2) 0.005 M EDTA solutions have only micellar structures.
Source: From Robinson, 1982 and Sweander et al., 1977.

by the filter-retained endotoxin is not overtly signaled. It becomes necessary, therefore, to ensure that additional LPS will not escape capture. Wickert (1993) discusses how this can be done. Two charge-modified filters are arranged in series separated by a testing port. When an assay, as by LAL testing, indicates exhaustion of the upstream filter, that filter is discarded. It is replaced by the downstream filter, which in turn is replaced by a new charge-modified filter. The LPS removal-capacity of the downstream filter is consumed as little as possible by this arrangement. This ensures against endotoxin breaking through. Alternatively, after replacing the consumed filter the flow can be reversed to pass first through it. In this manner, by timely testing, the second charge-modified filter in the series ensures the capture of whatever lipopolysaccharide may have permeated the first filter. Such filter replacement is practiced as frequently as necessary during the purification process (Fig. 3).

By contrast with the *hydrophilic* CA filter, the use of *hydrophobic* 0.2 μm rated inverse-phase (temperature-governed) polypropylene membranes could reduced the endotoxin concentration of solutions filtered through them by either mechanism. Utilizing a polypropylene membrane of 0.2 μm rating, the reduction attained an LRV of 1–3. The use of a 0.1 μm rated polypropylene membrane of similar inverse phase structure reduced the endotoxin by an LRV of 3–4. It would seem that the use of smaller pore size rated hydrophobic filters could utilize both sieve retention and hydrophobic adsorption in retaining the pyrogenic agent. Thus, the simultaneous removal of endotoxin by both hydrophobic adsorption and sieve retention can be ensured by the use of hydrophobic small pore-size rated membranes. The proper pore-sized microporous polypropylene membranes produced by the temperature-governed phase inversion process meet both requirements (Hiatt et al., 1985).

MODELS FOR ORGANISM ADSORPTIONS

The focus on protein adsorptions to filters may well be extended to parts of surfaces similarly agreeable to adsorptive bonding. Interspersed among different and varied

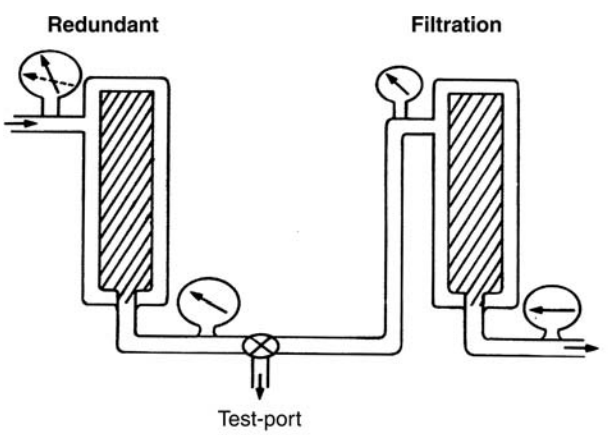


FIGURE 3 Two filters arranged in series separated by testing port. *Source*: From Jornitz, 2006.

molecular arrangements such areas, if sufficiently extensive and influential, should react accordingly in being conducive to hydrophobic adsorptions. This broadens our consideration to other phenomena usually not considered as involving adsorptions.

Molecular complexities characterize proteins, and also the polymeric structures of filters employed in sterilizing filtrations. Long chain structures of atomic constituents in varied arrangements are common to both. This allow for the simultaneous presence of different hydrophobic/hydrophilic groups or areas within large and complex molecular arrangements. This, it is speculated, may apply to the structures of organisms as well as to those of proteins. The bacterial cell surface consists of a peptidoglycan layer covalently linked to a variety of membrane proteins and anionic polymers. Although much of this surface is hydrophilic, some hydrophobic areas are simultaneously present as well (Zydney, 1996). Through hydrophobic adsorptions, those areas on the proteins join and bond with hydrophobic areas on filter surfaces (Dumitriu and Dumitriu-Medvichi, 1994). In the case of the proteins, the areas of hydrophobicity reflect that of the constituting amino acids. As stated, the most hydrophobic of which are tyrosine, phenylalanine, and tryptophane.

There is the hope in this writing that the experimental elucidations of protein adsorptions can serve as a guide to the better understanding of bacterial adsorptions. As expressed by Mittelman et al. (1998), and by Meltzer and Jornitz (2006), the molecules of proteins and of organisms are complex enough to present areas of both polar and non-polar character. In the similarity of their mechanism of retention by filters may lie a commonality of their management. Encouragement is given this expectation by the findings of Fletcher and Loeb (1979) showing that marine bacteria attach most readily to hydrophobic plastics. By contrast, the smallest number attach to negatively charged hydrophilic surfaces (Zydney, 1996, 440).

Organism Adsorption to RO Polymer

Encouragement is given the concept that organism attachments to filter surfaces may be imitative of those of proteins. Using radioisotopically labeled *Mycobacerium* BT2-4 cells, Ridgway et al. (1984a,b, 1985, 1986) studied biofilm formation on CA RO membranes. The adhesion of the organisms without the impress of a differential pressure was surprisingly rapid. It showed no log phase and was biphasic: An initial rapid adhesion, straight line with respect to time, was followed by a much slower rate of attachment, also linear with time. The first phase of adhesion took place over a 1–2 h period; the slower phase proceeded indefinitely. Typical saturation adsorption kinetics were involved in keeping with the Langmuir adsorption isotherm equation.

Colonization of CA membranes by the microbes was quite rapid, $3 \times 10^5 \text{cfu/cm}^2$ over a 3-day period. The greater degree of adhesion was to nylon (polyamide) membrane by 5- or 10-fold (Ridgway et al., 1984a,b).

The adsorption was interfered with by non-ionic surfactant. It is presumed that the surfactant molecules attach to the adsorptive sites of both organisms and membranes, and constitute a steric buffer between the hydrophobic ligands. Ridgway concludes from this that the adsorption phenomenon is hydrophobic in nature.

Interestingly, inactivation of the bacteria by monochloramine did not decrease their adsorption to the membrane surfaces. The implication is that the bacterial attachments are physicochemical rather than the result of such metabolic processes as exopolymer-mediated bridging of the electric double layer, or the result of chemotactic responses. In any case, the different polymeric materials adsorbed organisms to different extents.

The individuality of an organism with regard to the different polymers is mirrored by that of a given polymeric material for different organisms. Thus, *Mycobacterium*

adheres 25 times greater than does a wild strain of *Escherichia coli*. The adsorption of *E. coli* and *Mycobacterium* correlates with their surface hydrophobicity; similarly, for hydrophilic *Acinetobacter phosphadevorus* and a more hydrophobic strain.

Biofilm Adhesions to Surfaces

Figure 4 illustrates the extents to which different polymeric membranes adsorb certain proteins. Each polymeric material has its own individual propensity for adsorptions; as also that given the same polymer, different proteins adsorb to different extents.

This may have significance to bacterial fouling. The attachment of organisms to surfaces, often in the form of biofilms, is a major problem in many situations, and has, accordingly, also been widely studied (Characklis, 1990; Ridgway, 1987; Mittelman et al., 1998; Meltzer and Jornitz, 2006). It is a considerable stretch to equate protein adsorption to biofilm formation. However, to the extent that areas of proteinaceous compositions may characterize organisms, they may react equivalently. Surface heterogeneity implies such independence of action regardless of the character of the remainder of the surface of which they are part. Such serves to justify this speculation. Ridgway (1986a,b) in Figure 5 shows the different extents to which a *Micobacterium* adsorbs to a polyamide RO filter, and to one composed of CA. The polyamide adsorbs much more *Micobacteria*.

Biofilm Similarities to Protein Adsorption

It can be hypothesized that the formation of biofilms involves the very mechanism that characterizes protein-to-filter attachments. Biofilms are formed by planktonic organisms attaching to the solid surfaces. It is speculated that the organisms in adhering to the solid surfaces may utilize as an adhesive an extracellular polymeric substances produced by them. Alternatively, the adsorptive attachments may result from the influence of the electric double layer. Indications of hydrophobic adsorptions in biofilm formation has been suggested. More importantly, reductions in free surface energy are involved in

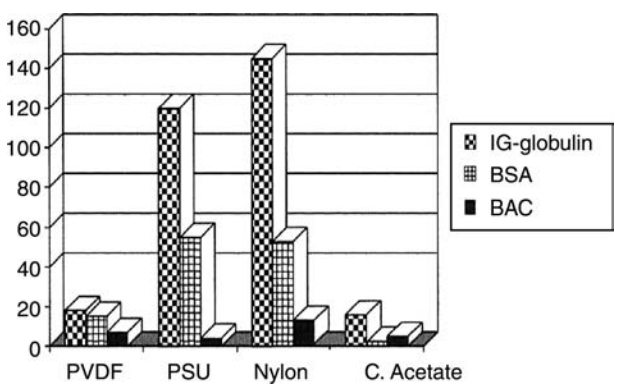


FIGURE 4 Non-specific adsorption of different membranes. *Source*: From Truskey et al., 1987; Marshall, 1992.

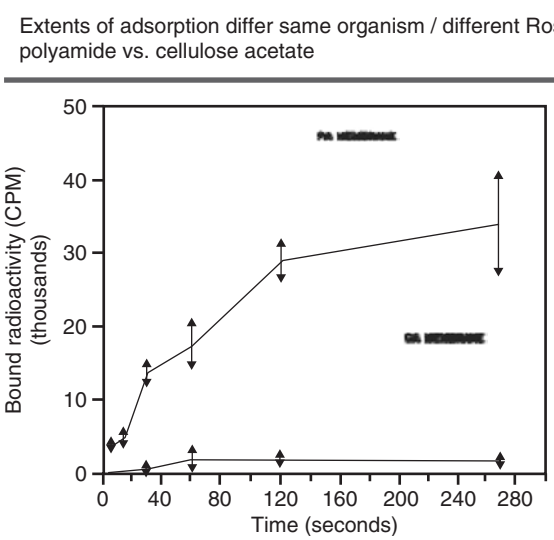


FIGURE 5 *Upper line*: Micobacterium adsorbed to polyamide RO membrane. *Lower line*: Micobacterium adsorbed to cellulose acetate RO membrane. *Source*: From Ridgway et al., 1984a;

the attachments. This latter quality is a result, if not a cause, of hydrophobic adsorptions, as will be discussed. From the foregoing, it seems there is a degree of commonality between biofilm formation and protein adsorption.

The adsorption of organisms from their suspensions onto solid surfaces would involve the same forces of attraction and repulsion that regulate the adsorptive joining of one colloidal particle's surface to that of another. The process involving organisms would manifest itself in their adsorptive retention to the filter surfaces.

Marshall (1992) points out that bacteria, as also particles in general, are kept from contact with surfaces by electrostatic forces. This includes the surfaces of other organisms. The repulsive forces involved are effective at long range, and prevail over the weaker attractive forces that are simultaneously present. As will be described in the forthcoming discussion of the electric double layer, the repulsions are attenuated by higher electrolyte concentrations. The attractive forces, albeit weaker, can then assert their influence. The adsorptive interactions that follow would result in the formation of a biofilm.

Fuoss Effect

In suggesting a similarity between protein adsorptions to polymeric filter surfaces and organism attachments to the same type surfaces, it is speculated that the term "adsorption" as used in the phrase "protein adsorption" includes a more varied expression of the same mechanisms that fixedly bond other surfaces to one another. The bonding to activated carbon of organic molecular substances, whether in the form of vapors, liquids or particles, as utilized in gas masks or in water purification contexts, is a classical example of adsorption. As will be seen, the ionic strengths of the aqueous solutions from which proteins may be adsorbed also govern the adsorptive bonding of the organics to the surfaces of the activated carbon particles. In this instance as well, there is an apparent similarity in cause and effect. It is speculated that the adsorption of organic compounds including proteins to activated carbon surfaces is part of a like operation.

The positive effect of ionic strengths on adsorptive sequestrations is forthcoming from the field of water treatment and involves the adsorption of organic substances by

activated carbon. This is greatly enhanced by the presence of calcium and magnesium ions. According to Weber et al. (1983), the adsorption of humic materials by activated carbon is pH-dependent and is influenced by the presence of inorganic ions in the solution (Fig. 6). Calcium is slightly more effective than magnesium, and divalent ions are more influential than monovalent ions by an order of magnitude; potassium ions are slightly more effective than sodium ions. The salutary effects of lower pH on increasing adsorption had previously been remarked upon by Schnitzer and Kodama (1966).

A plausible explanation may derive from the Fuoss effect as discussed by Ong and Bisque (1966) and as advanced by Ghosh and Schnitzer (1979). The Fuoss effect states that large polymeric electrolytes, such as derive from humic acids, exist in solution in a coiled configuration, as indeed do all polymers. Polymeric molecules, as also protein structures, increasingly unwind and extend themselves the diluter the solutions. Higher ionic strengths, to the contrary, promote the tightness of such coilings. The contractions of the humic acid molecules under the influence of higher ionic strengths has two adsorption-promoting consequences. The size of the polymer molecules decreases as they become progressively more coiled. In the process, the folding of the polymeric chains increasingly confine their hydrophilic moieties, and more openly present their hydrophobic amino acid constituents, of which tryptophane, tyrosin, and phenylalanine are the most extreme. The first effect further increases the ease of interstice penetration; the second promotes hydrophobic adsorptions. Thus, the presence of ions such as hydronium, ($H–H_2O^+$ or $H_3{}^+O$), calcium, and magnesium promote the molecular folding. Both the capacity for adsorption and the rate of adsorption are increased. The adsorption is rate-dependent.

Evidence that the adsorption onto active carbon surfaces of organic materials derived from humic substances is promoted by lower pHs was furnished by Weber et al. (1983) and by Schnitzer and Kodama (1966) and was stated also by Michaud (1988). Weber et al. (1983) found that the adsorption isotherm for humic acid on an activated carbon, although increased somewhat by going from pH 9.0–7.0, increases markedly when the pH is lowered to 3.5. As will be discussed, lower pH values, equivalents to higher hydronium ion concentrations, also promote the adsorption of proteins to hydrophilic filter surfaces.

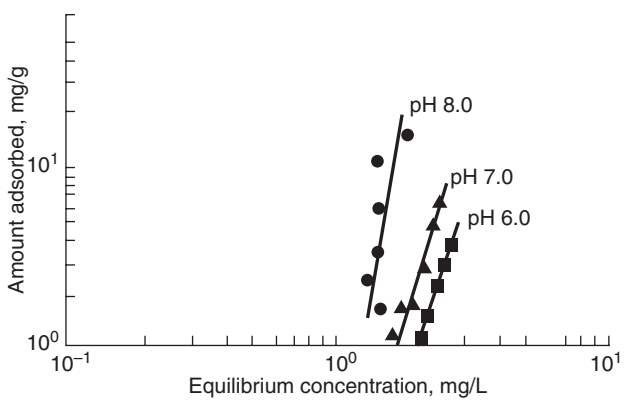


FIGURE 6 Adsorption isotherms for humic acid on carbon-effect of pH. *Source*: From Weber, 1983.

Qualitative Measurement of Protein Binding

That specific proteins exhibit differences in their adsorptive proclivities has been remarked upon. The usefulness of a means of measuring this tendency is obvious. Badenhop et al. (1970) have devised and utilized such a test method. A standardized drop of an aqueous solution of serum albumin was placed upon the surface of each of three membrane filters. The extent to which the water-drop spread was evidenced by the area of wetness. This was essentially the same for the three filters: cellulose triacetate, cellulose nitrate, and an experimental PVC. However, the spread of the albumin within the wet area differed. The visual detection of the albumin was made possible by the use of Ponceau S stain. It was found that the albumin spread along with the water over the cellulose triacetate when the bovine albumin concentration was 100 mg percent. At that concentration level, the albumin spread only very little on the cellulose nitrate and even less on the experimental PVC. At a concentration of 700-mg percent, the spread of albumin over the experimental PVC filter did not increase much, but it did so for the cellulose nitrate. This is interpreted as indicating the relative binding forces of the three polymers for serum albumin in aqueous solution.

The cellulose triacetate is seen as exerting the least interaction. Its saturation point is satisfied with a small amount of albumin, leaving enough to spread and adsorb to larger areas. Thus, it is the least adsorbing and the least interfering in the albumin's spreading along the membrane surface. The experimental PVC by this measure adsorbs the most strongly of the three polymers examined. It adsorbs so strongly and to such an extent that it serves totally to saturate the contact spot. None is left available for spreading even at the 700-mg-percent level.

The nitrocellulose binds albumin strongly, but its attractive forces at a given area are not as avid as those of PVC in attaining saturation. Thus, at the 700-mg-percent level, unlike the PVC, some of the albumin remains to spread further.

The adsorptive strength of a membrane is, thus, expressible in terms of its adsorption to a specific protein, both in terms of the quantity of protein it binds to the point of saturation, and in the extent available for adsorption beyond the point of contact.

Hydrophilization of Membranes

Protein studies have generally shown that filters composed of polar structures, such as cellulose triacetate, exhibit minimal tendencies towards adsorption. (Nitrocellulose membranes, as stated, disclose a higher tendency towards adsorption, possibly through the agency of hydrogen bonding.) Inherently hydrophobic filters, such as an experimental PVC, manifest the highest proclivity of these filter types to interact with proteins. In an effort to minimize the fouling of filters by protein depositions, hydrophobic filters generally available from filter manufacturers have been modified by chemical grafting to convert them to filters characterized by hydrophilic surfaces. The methods by which the "hydrophilizing" alteration is managed are largely proprietary. It is known, however, that methyl methacrylate, probably under free radical attack, can react with given molecular structures to introduce polar ester groups onto the otherwise hydrophobic substrate surface. The reaction is catalyzed by cobalt 60 gamma emanations. The heretofore hydrophobic filter, liable to fouling by proteins, is essentially prevented from so being by conversion of its surface to one that is hydrophilic.

Kim et al. (1994) modified the surface of a polyethylene membrane by introducing 5–7 mol alcoholic hydroxyl group per kilogram; enough to cover the entire polyethylene

surface. The filter's saturation capacity for bovine λ-globulin was reduced from 7 mg/m^2 to under 1 mg/m^2 as a result of the filter's surface having been hydrophilized. The change in surface character was from non-polar and hydrophobic to hydrophilic. The difference in the adsorptive response resulted from the presence of the O–H atomic couple that characterizes alcohols. The oxygen atom of that atomic pair in its acquisitive and unequal sharing of the bonding electrons with the partnering hydrogen atom assumes a partial negative electric sign. The partial loss of the bonding electrons by the hydrogen atom leaves it with a partial but positive electrical charge. It is this partial-charge that transforms what would otherwise have been a hydrophobic bonding into one that is dependent on the attraction of opposite electrical signs, albeit only of partial electrical potential.

That the hydrophilization of membranes composed of hydrophobic polymers makes them less receptive to protein adsorption is a relatively recent understanding within the pharmaceutical industry. However, it was demonstrated over three decades ago by filtration practitioners in the brewing industry. Badenhop et al. (1970) showed that coating an hydrophobic experimental PVC membrane with a hydrophilic coating resulted in a significant increase in the filtrative throughput of heavy beers. This resulted from a reduction in the protein fouling of the filters (Table 2). This argues for an awareness of solutions to problems that may be forthcoming from applications outside of the pharmaceutical industry.

In experiments with filtering heavy beers, Badenhop et al. (1970) found that the throughputs were reciprocal to the adsorption tendency. The greater the adsorptive effect, the more the "fouling" of the filter and the smaller the throughput. For proteins in general, the fouling effect, the interference with flow, depends upon the relative sizes of the protein molecule and of the filter's pores.

Competitive Adsorptions

Certain agents competitively preempt the adsorptive sites to block the adsorption of other molecules. This blocking effect is particularly noticed in protein work. Hawker and Hawker (1975) found that gamma globulin is retained in smaller amounts than is fibrinogen. The same action adsorbed IgG from antisera, as measured by nephelometry. Interestingly, addition of polyethylene glycol to the antisera or polyethylene glycol prewashing of the filters prevented such losses. As stated, such shielding effects, ascribed to the preferential adsorption of the polyethylene glycol to the membrane and to consequent blockage of the adsorptive sites, have been used in electrophoresis work. The membrane strip used in electrophoresis may be coated with various wetting agents or with

TABLE 2 Comparison of Uncoated and Hydrophilic-Coated PVC-Type Membranes in Heavy-Beer Filtration

Beer A	
Experimental PVC	175
Hydrophilic coated	263
Beer B	
Experimental PVC	241
Hydrophilic coated	720

Source: From Badenhop et al., 1970.

albumin solution, etc. The serum proteins being electrophoresed plate out in different spread patterns than they do on the untreated strip.

Surfactants are particularly efficient at preempting the adsorptive sites and at blocking them from other type molecules. This can be considered as resulting from a competitive action that favors the stronger and, thus, the more stable bonding.

Surfactants: Adsorption and Its Blocking

Wrasidio and Mysels' (1984) findings that the nonionic surfactant rinsing (with Triton X-100) of polystyrene latex spheres renders their retention less complete by conventional 0.2 μm rated membrane was taken to indicate adsorptive influences. Hawker and Hawker (1975) show that membrane treatment with a nonionic surfactant (Tween 20) rinse presumably similarly blocks adsorptions. Prewashing with dilute plasma has almost the same effect. However, the adsorption site-blocking adsorbed plasma leaves some charged protein bound within the filter. This serves to attract and bind additional protein. Thus, prewashing with protein is somewhat less effective than Tween 20 at preventing fibrinogen retention by 0.2 μm rated membranes (Hawker and Hawker, 1975). Pitt et al. (1985) showed that various membranes adsorbed proteins differently and that the same polymeric material, possibly influenced by a different wetting agent, yielded disparate results.

The wetting of hydrophobic surfaces by aqueous media is made possible by the mediation of surfactants. However, the effect is quantitatively unreliable unless complete wetting takes place. In less than perfect wetting large coefficient of variation result. Liquids less polar than water, e.g., isopropanol, are less influenced by the presence or absence of surfactant. Polymeric surfaces are characterized by critical surface tension (CST) values. Liquids lower in surface tension, (CST), will wet them; those higher will not. For example, polyethylene has a CST of 32 dyn/cm. Water with a surface tension of 72 dyn/cm will not wet the polymer. The addition of surfactant lowers the liquid's surface tension to where wetting can occur. Not surprisingly, Emory et al. (1993) found that different surfactants differed in their wetting effects.

Of greater interest, the use of surfactant in conjunction with latex particles affects their retention. It minimizes the adsorption effects and counters particle flocculation (Emory et al., 1993). These experimenters investigated the effects of three different types of surfactants–nonionic, anionic and cationic – on the retention of latex particles by each of four types of membranes. The membranes tested were of polysulfone, nylon 66 (aka, nylon 6.6), hydrophilic polyvinylidene fluoride (PVDF) and mixed esters of cellulose, the latter free of its usual wetting agent content.

For all the surfactants, the retention of the 0.40-m beads by the 0.45-m-rated membranes decreased asymptotically with surfactant concentration. This can be taken as an index of the blocking of the adsorptive sites by preemptive surfactant adsorption (Fig. 7). The surfactants tested gave somewhat different retentions for the several membranes. Generally, the anionic surfactant yielded lower retentions, the cationic higher; that is the cationic blocked more than did the anionic. In the case of the nylon 66 filter, the nonionic and cationic agents produced the least retention, the most blockage. Conclusions regarding comparisons among the filter types tested must be tempered by the knowledge that while all were 0.45-m-rated, no common rating standard exists. The different filters might possibly have received different ratings had they all been characterized by a single method. Variations in the retention effect could conceivably reflect actual differences in the pore sizes.

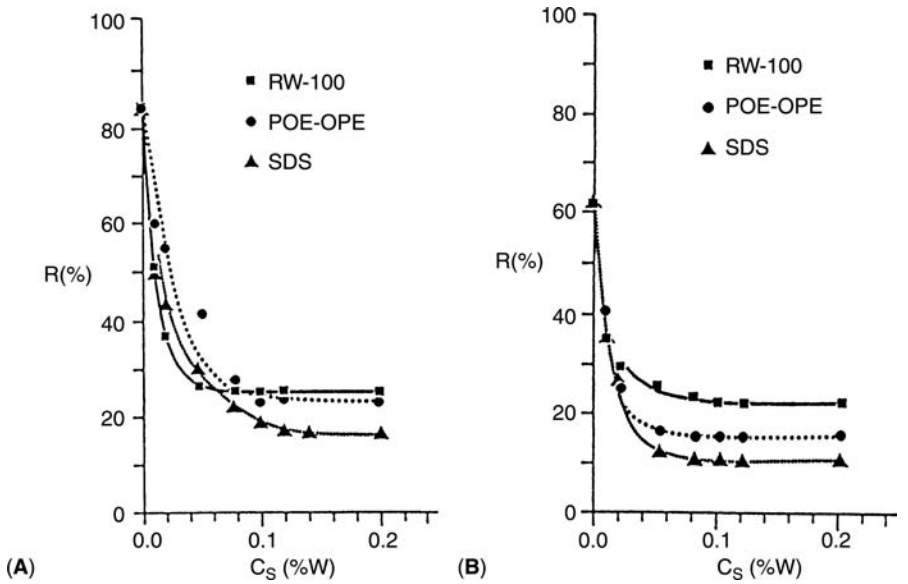


FIGURE 7 Retention (R) of 0.40 μm latex particles of (**A**) 0.45 μm modified PVDF membrane filter and (**B**) 0.45μm mixed cellulose esters membrane filter as a function of surfactant concentration for a particle feed concentration of 10^8 particles/mL. *Source*: From Emory et al., 1993.

The effect of wetting agents (surfactants) on bubble points is to lower their values by way of reducing the surface tension of the liquid medium. Certain membranes are manufactured using wetting agents in order to avoid occasional integrity test failures caused by the borderline wettability of the polymer itself. Certain polysulfones are prepared using a Klucel surfactant, an ethylene oxide adduct of a low molecular weight cellulose, the etheryl oxygens and hydroxyl groups thereof invite the hydrogen bonding (wetting) with water molecules. This or similar wetting compounds such as Triton X-100, had traditionally been added to compositions of mixed esters of cellulose. Polyhydroxy compounds that invite wetting water have a similar effect. Thus, CA membranes sometimes contain glycerin whose wetting action derives from the three hydroxyl groups of its molecule structure. The Tritons, adducts of ethylene oxide chains to alkylated aryl phenols, contain repeating etheryl oxygen units each of whose two pairs of unshared electrons hydrogen bond with water molecules to produce the desired wetting action. The Tritons, having been shown to be cytotoxic (Cahn, 1967), are eschewed in tissue culture work. Other surfactants may perhaps become substituted for them. It should not be taken for granted that the designation of a filter's identity solely in terms of its polymeric composition necessarily implies an absence of wetting aid and of its effect on extractables. Relevant information should be provided by the membrane manufacturer.

Competitive Adsorptions

Large molecules may be complex enough to simultaneously contain atomic groupings that are hydrophilic along with those that are hydrophobic, as determined by the polarity and extent of their various substituent groups.

Proteins are generally seen to undergo hydrophobic adsorptions. It seems logical to assume that the more hydrophobic the protein and the adsorptive site, the stronger the

hydrophobic bonding. An hierarchy of bonding strengths should result from the possible mixes of hydrophilic and hydrophobic sites that differentiate among protein structures.

The latex particles consist of polystyrene cross-linked under free radical catalysis with divinylbenzene. They are intrinsically non-polar, free of partial-charge substituents. Thus, they are highly hydrophobic. They, therefore, undergo hydrophobic adsorptions in competiton with proteins or surfactants of similar tendencies. In their adsorptive advantage over less hydrophobic, more hydrophilic molecular structures, the latex heads pre-empt the hydrophobic adsorptive sites, reducing or preventing the filter from bonding to protein, surfactant, or other less hydrophobic (more hydrophilic) molecular entities.

In the case of the surfactants, the more hydrophobic the molecule, the more thorough its adsorptive bonding, and the stronger its competitive interference in blocking the adsorption of proteins that are more hydrophilic. The more polar the protein, the more easily it is blocked from adsorptive uptakes, pre-empted by less hydrophilic surfactants and more hydrophobic latex particles.

Surfactants owe their detergent action to their structural bifunctionality. Part of their molecules are free of polarity, often composed of alkylated aromatic structures, while the remaining portion, often a polyether chain, is marked by partial-charged oxygen atoms that, being hydrophilic, bond with water molecules.

The singularity of a given adsorptive bond reflects its individual placement in the gamut of hydrophilic/ hydrophobic molecular mixes that define the types and strengths of the competitive, adsorptive interactions. This complexity is multiplied by the various shades of hydrophilicity and hydrophobycity that can characterize the molecular architecture of the filters.

MECHANISMS OF ADSORPTION

Hydrophobic Adsorptions

Given its abstruse nature, it is not surprising that there are several explanations for the phenomenon of hydrophobic adsorption. No challenges are posed to its being a reality, but there are differences concerning its cause and effects. Adamson (1982) writes, “the term ‘hydrophobic bonding’ is appropriate to conditions wherein there is an enhanced attraction between two surfaces (as of a particle and filter) exposed to a liquid if the liquid–particle interaction is weaker than the liquid–liquid interaction.” The term “hydrophobic” implies an antipathy for water. It is demonstrated by an extreme immiscibility with water. This derives from an absence of polar groups capable of hydrogen bonding to water by way of electrical partial-charge interactions. Hydrophobic adsorption is, therefore, believed by some authorities to be *free* of partial-charge involvements. As will shortly be seen, the driving force of the adsorption phenomenon is believed by these authorities to result from diminutions in free surface energy that accompany such adsorptions, as explained below.

The rebuttal of this hypothesis holds that electric charges *are* involved, although in a less than obvious fashion. The more apparent electric double layer actions involving electrical charges do arise from molecular structures, such as ester, and carboxylic groups that contain oxygen atoms. They do give rise to dipoles on account of the strong electro-negativity of their oxygen atoms. The dipole–dipole and other electrical interactions account for the attractions between solid surface sites that result in adsorptive sequestrations, and also colloidal agglomerations. However, although such polar features are absent from hydrocarbon molecules, the latter, nevertheless, do interact in the manner

that suggests adsorptive influences based on electrical charges. The apparent contradiction requires clarification.

The VDW forces that exercise attracting interactions among hydrocarbons bereft of oxygen atoms or other polarizing features are hypothesized as being due to "instantaneous non-zero dipole moments" that result in attractions, albeit weak ones. This would explain the hydrocarbon interaction as also being charge related. In this view, hydrocarbon molecules, here taken as the archetypical non-polar substances, are motivated by the opposite signs of their VDW type partial-charges to adsorptively connect with other hydrophobic molecules in hydrophobic adsorptions.

The simpler hypothesis that does not rely upon charge interactions between hydrocarbon molecules is possible. In agreement with time-honored alchemists' observations, namely, "like prefers like." It is accepted that hydrocarbon molecules do tend to connect with other hydrophobic molecules. The implication is that the hydrocarbon molecules' VDW attraction of the one for another is the key driver in hydrophobic adsorptions.

This seems also to be the view of Fletcher (1996:3). He sees the water adsorbed to the bacterium and filter surfaces as a (separating) barrier to their coming together. Moreover, removal of the water is "energetically unfavorable." However, if either surface has non-polar groups or patches the resulting hydrophobic interaction will displace the water and allow a closer approach of the two surfaces. This more orthodox explanation does not rely upon charge interactions between hydrocarbon molecules. It cites the reduction in free surface energy that is an accompaniment of hydrophobic adsorptions. The implication is that this is the force that motivates the coalescence of the dispersed hydrophobic phase.

Nevertheless, the gathering of the hydrophobic material and its separation from the water is more distinctly explained by Tanford (1980) who quotes Hartley as stating, "The antipathy of the paraffin-chain for water is, however, frequently misunderstood. There is no question of actual repulsion between individual water molecules and paraffin chains, nor is there any very strong attraction of paraffin chains for one another. There is, however, a very strong attraction of water molecules for one another in comparison with which the paraffin-paraffin or paraffin-water attractions are very slight." Thus, Tanford (1980:Chap. 5) expostulates that it is the water molecules' alliances among themselves that rejects interactions with the hydrocarbon molecules, causing a concentration of the latter: "The free energy is representative of the attraction between the substances involved. The free energy of attraction between water and hexane or water and octane obtained at 25°C is about –40 erg/cm^2 of contact area; the free energy of attraction of the hydrocarbons for themselves at the same temperature is also about –40 erg/cm^2; but the free energy of attraction of water for itself is –144 erg/ cm^2. It is clearly the latter alone that leads to a thermodynamic preference for elimination of hydrocarbon–water contacts; the attraction of the hydrocarbon for itself is essentially the same as its attraction for water." The driving force of the hydrophobic adsorptions, then, is the reduction in Gibbs' free surface energy that results from the strong mutual attractive forces that manifest themselves in hydrogen bonding among the water molecules. Partial-charge influences need not be involved.

At this point we are obliged to briefly consider the hydrogen bond; covalent bonding; partial-charges; and the electric double layer. A fuller treatment will follow subsequently.

The water molecule is tetrahedral in shape. Each of its corners holds either a pair of electrons or a hydrogen atom. Each of the partly positive hydrogen atoms of one water molecule can form a hydrogen bond with a partly negative oxygen atom of each of four different water molecule, etc. Actually, two or three is the usual number. This process, repeated throughout the water volume, in effect creates an (imperfect) interconnected

network. Thus, the molecules of water in its solid state (ice) exist as tetrahedral hydrogen bonded structures. Much of this ordered form persists even in the mobile liquid.

The hydrocarbon molecules with little affinity for the water molecules are rebuffed from intruding among these spatially, tetrahedrally-ordered arrangements. It is the network formed by the water molecules among themselves that in *expelling* the hydrocarbon molecules causes their segregation. These may conjoin also to the hydrophobic areas of solid surfaces they encounter; such as of pipes or filters. In their coming together, the hydrocarbon molecules, as also the water molecules, effect a reduction in the total free surface energy. It is likely that micellar groupings are involved under the influence of area-minimizing forces.

With regard to hydrophobic adsorptions to filter membrane surfaces, Zydney's (1996) view seems closer to that of Fletcher (1996). He describes the phenomenon as follows: "solute-membrane interactions can occur only if sufficient energy is provided to displace the H-bonded water molecules from the surface of both the membrane and the macrosolute (protein). In contrast, the removal of unbonded water molecules from the surface of a hydrophobic membrane or macrosolute is energetically very favorable, leading to a very strong 'attractive' interaction between hydrophobic surfaces in aqueous solutions." Although referred to variously as hydrophobic adsorption, or hydrophobic bonding, "it really reflects the change in energy or entropy due to the dehydration of the two surfaces, and the formation of additional hydrogen bonds among water molecules."

Significance of Solubility

It is possible to generalize regarding the adsorption of materials from aqueous media by viewing the adsorptive phenomenon as being in competition with the tendency of the material to remain in solution. Solublization involves partial-charge interactions between the water molecules and the molecules being brought into solution. This is the same type bonding that results in the hydration of ions. The adsorptive union is between the full charge on the ion and the partial-charge of opposite sign on the atoms of the water molecule. The less extensive the bonding with water, the less water-soluble the material; that is, the smaller the interaction between the given molecular entity and water, the easier it is to remove it from solution by hydrophobic adsorptive interaction. By this measure, less ionized or non-polar molecules are easier to adsorb because they have less affinity for the water molecules than the water molecules have for themselves. Hydrophobic adsorption assumes its importance as a particle retention mechanism on the basis of the relative strengths of the several interactions that are possible in a given situation, namely: particle to particle; water to particle; and water to water; with the last being the strongest.

Free Surface Energy

A proper treatment of the subject requires the application of thermodynamics. An effort is being made here to set forth the concept of free surface energy in a less rigorous manner. The beneficiaries will be those who, like the authors, are limited in their ability to probe the occult and mysterious.

Consider a droplet of water. Every water molecule within its depth will interact with its neighbors to the full extent of its powers to undergo bonding. As a result, a given volume of water will, in effect, consist of a network of water molecules connected to one another by hydrogen bonds. The propensity of the water molecules to bond is a measure of the energy available to them for interaction with other (suitable type) molecules.

However, the water molecules situated on the spherical outside boundaries of the water droplet and constituting its interface with non-water molecules have no neighbors on the droplet's periphery suitable for bonding. The unexpended energy remaining in their non-bonded region is the free surface energy being discussed. Liquid water dispersed in discrete droplet form has a proportionally large surface area; and, therefore, a large total free surface energy. The energy expenditure undergone in bonding interactions serves to coalesce the droplets into larger volumes with a consequent diminishment in total surface area. A loss in the total free surface energy is the result.

The Electrical Double Layers and Partial Charges

Adsorptive effects can be explained by considering the electrical aspects of surface chemistry (Adamson, 1982: Chap. V). Largely, these form the basis of the Debye–Hückel theory, contributed to and enlarged upon by notable authorities in the field. It is sufficiently recondite in its mathematics to frustrate its presentation by the authors of this chapter except as a generality.

As a prelude to explaining the electrical double layer it may be timely to review the nature of the partial-charges that are commonly considered to be of pertinence in the formation of adsorptive bonding interactions.

Adsorptive Forces

Atoms in their neutral state are each characterized by a set number of electrons. In interacting with one another to form various molecules, two atoms become mutually bonded by strong electrical forces. These result from one atom acquiring an electron from the other. The electron is conventionally regarded as bearing a negative electrical sign. Thus, the atom that accepts the electron is said to be negatively charged (–), since it now has one electron (negatively charged) more than it has in its neutral state. The recipient atom in donating its electron departs from its neutral status by losing an amount of negative electricity; namely, an electron. It thus becomes plus charged. The two partnering atoms sharing in this electron- exchange are said to be chemically bonded; united by the attraction that oppositely electrical charges have for one another. This, the ionic bond, is the product of an electron being transferred from one atom to another.

Covalent Bonding

The two electrons that commonly constitute a covalent chemical bond are each donated by one of the interacting atoms. However, the two bonding electrons are not shared equally. An atom, as a consequence of its size, may draw the electron pair closer to itself and so acquires a greater portion of the negative charge. Accordingly, the other bonding atom, bereft of a portion of the negative charge, in effect becomes plus charged. It is from such unequal sharing of the bonding electrons that the partial-charges originate. The partial-charge on an atom of one molecule will tend to connect with (adsorb onto) an oppositely charged atom on another molecule. The hydrogen bond is an example of such an interaction.

Hydrogen Bond

Aspects of the hydrogen bond were discussed above. The hydrogen bond arises from a dipole–dipole interaction (Fig. 8). It is the most important of such interactions. The water

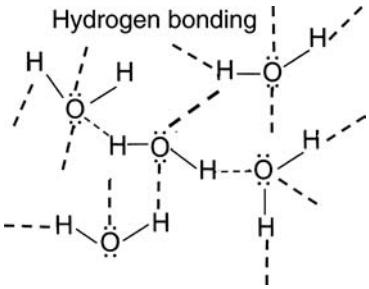


FIGURE 8 The hydrogen bond. *Source*: Courtesy of Capitola Presentations.

molecule, H_2O, consists of two hydrogen atoms each bonded to the same oxygen atom. The nucleus of the oxygen atom pulls the bonding electrons more strongly to itself and away from the hydrogen atoms. The bonding is not disrupted, but the bonding elements become partially charged. The unequal sharing of the electrons makes the electron-richer oxygen partially negative, and the proportionately deprived hydrogen atoms partially positive. This creates the O^-H^+dipole. (Tanford, 1980:Chap 5). There are two hydrogen atoms originating from two different water molecules that connect to a single oxygen atom of one of those molecules. One hydrogen atom is of the pair chemically bonded to the oxygen atom to comprise the water molecule. The other forms the hydrogen bond that bridges one water molecule to the other. Interestingly, the two hydrogen atoms are not equidistant from the oxygen atom. The one attached by valence forces to the oxygen atom is at a distance of 1.00 Å from it. The H-bonded hydrogen is 1.76 Å apart from that oxygen. The oxygen atoms of the two interacting water molecules are 2.76 Å apart, while the two nearest non-hydrogen bonded oxygen atoms are 4.5 Å distant from one another. The chemically bonded hydrogen is more closely attached; perhaps an indication of the relative strengths of the two types of bonds.

The attractive forces of the oppositely signed partial-charges decrease rapidly with the distance between the dipoles. Only the proton (hydrogen atom, of atomic weight 1) is small enough to approach the electronegative oxygen atom closely enough to establish the H-bond. Moreover, the electromagnetic associations involving hydrogen atoms in dipole arrangements are only strong enough to be formed with the most electronegative elements, namely, fluorine, oxygen, and nitrogen in decreasing order. Nevertheless, the H-bond, although weak in its energy of attraction, figures significantly in many fields of chemistry, and has importance especially in protein chemistry.

As stated, the water molecule is tetrahedral in shape. In its solid (ice) state it exist as tetrahedral hydrogen bonded structures. Much of this ordered form persists even in the mobile liquid. Each of the tetrahedral corners holds either a pair of electrons or an hydrogen atom. Each of the partly positive hydrogen atoms of one water molecule can form a hydrogen bond with a partly negative oxygen atom of each of two different water molecule. This process, repeated throughout the water volume, creates an interconnected molecular network that includes H-bonded rings and chains. The network of H-bonded water molecules requires the heat energy of boiling water to break the adsorptive bonding to set free the individual water molecules in vapor form, namely, steam. Interestingly, dimers of the water molecule exist to some extent even in steam; so strong is the hydrogen bond.

The bond strengths of partial-charges are less than those of the fuller electrical forces that characterize the chemical bonds. They are sometimes referred to as physical bonds. It is largely this type bonding that motivates the adsorptive interactions among the partially-charged atom of different signs sited on different molecules.

Hydrogen Bonding Interactions

That proteins tend to adhere to hydrophobic surfaces by way of the hydrophobic adsorption mechanism seems an established fact. In agreement with that explanation, studies on protein fouling of filters have generally shown that filters composed of polar structures, such as cellulose triacetate, exhibit minimal tendencies towards protein adsorptions. Hydrophobic adsorption is, however, not the only mechanism for such attachments. Thus, nitrocellulose membranes disclose a higher tendency towards protein adsorption, possibly through the agency of hydrogen bonding.

The efficacy of hydrophilization has not been restricted to pharmaceuticals. This practice has been put to good use in certain other applications including one considered by many to be of high purpose. Hydrophilization ameliorates the filter clogging (fouling) caused by the interactions of the filter polymer with the protein (albumin) present in beers. Larger production throughputs of the beverage are gained from hydrophilization of the filter (Table 3).

As Table 4 illustrates, filters of nitrocellulose, a polymeric organic ester whose structure contains three of either or both of hydroxyl or nitro substituents, interacts with beer proteins with considerable avidity. The mechanism of the intermolecular attraction is believed to result from hydrogen bonding between the partial negatively charged atoms of the nitro groups and the partial positively charged hydrogen atoms of the protein molecule. However, by contrast, the throughput of beer utilizing an experimental Dynel microporous membrane was even more restrictive (Table 4). Dynel, being a copolymer of vinyl chloride and acrylonitrile, is strongly hydrophobic. It likely favored protein adsorption though the exercise of its hydrophobicity, rather than through hydrogen bonding although its nitrogen atoms are amenable to hydrogen bonding. Hydrophobic filters, such as the experimental PVC membrane previously mentioned, manifest the highest proclivity of these filter types to interact with the proteins present in beers.

Electrophoresis

The proteins present in normal human serum can be separated by electrophoresis into a pattern of some seven distinct areas. A rectangular (2×4 inch^2) section of microporous

TABLE 3 Comparison of Heavy-Beer Throughput for 0.8-μ Nitrocellulose and Dynel Membranes

	Nitrocellulose	Dynel
	1.44	1.05
	1.55	1.25
	1.18	0.89
	1.30	1.23
	1.24	.99
	1.51	.74
Average	**1.37**	**1.03**

Filtration results are given in total liters throughput per cm^2 of filter surface area in a constant-flow-rate (30 ml/min/cm^2) filtration with a 60-psig cutoff point.
Source: From Badenhop et al., 1970.

TABLE 4 Flu-Vaccine Filtration Volume in MLS/Seconds

0.45 μm Mixed cellulose esters			Mixed cellulose esters		
Manuf. I			Manuf. II		
36/90	38/90	33/90	28/90	25/90	30/90
38/120	41/120	34/120	31/120	27/120	34/120
(Titers 64, 65%)			(Titers 64, 65%)		
0.45 μm Cellulose triacetate			0.45 μm Dynel-type		
40/90	46/90	40/90	64/90	48/90	52/90
42/120	50/120	42/120	70/120	53/120	57/120
45/180	55/180	43/150	78/180	57/180	63/180
	58/210		80/210		
(Titers 90, 91, 91%)			(Titers 89, 87%)		

Source: From Tarry and Meltzer, 1978.

membrane is soaked in a buffer medium capable of conducting an electric current. A thin line of serum is deposited transversally on the membrane. The imposition of an electric current causes the migration of the serum proteins towards an electrode. They separate into some seven bands in accord with their molecular weights and charge densities (Fig. 9). The pattern common to normal health is known. Abnormal results are subject to diagnostic interpretations by pathologists.

The technique involves a competition between the fixed adsorptive bonding of protein to the polymer matrix, and the motivation supplied by the electric current. It is found that cellulose diacetate membrane permits the protein migration into the expected pattern. However, cellulose nitrate membrane so strongly bonds the protein to the area of its deposition that even increasing the current is of no avail. Ultimately the heat engendered by the resistance to passage of the current, in accord with Ohm's Law, denatures the serum proteins into an undifferentiated deposit (Fig. 9). These results indicate the relative appetites of the two membrane polymers towards serum protein adsorptions (Gebott et al., 1969).

Vaccine Adsorption to Filters

As shown in Table 4 the filtration of influenza vaccine through microporous polymeric membranes results in the loss of titer, a costly and undesirable consequence of vaccine purification by filtration. The loss of titer with a mixed ester of cellulose membrane was 35%; with CA it was 9%. A membrane composed of vinyl chloride acrylonitrile copolymer lost 11% of titer. Moreover, the effect on the filter in terms of throughput was significant. The CA filter in 47-mm disc form yielded an average of 38 mL over a 120-sec interval; the copolymer produced 80 mL over 210 sec; and the mixed esters gave 58 mL in a period of 210 sec. The influence of the polymeric composition of the filter is evident (Tanny and Meltzer, 1978).

It is essential to test different filter membrane polymers in the initial stages (commonly pre-clinical phase) to evaluate not only throughput, but especially unspecific adsorption. Most commonly, unspecific adsorption is responsible for excessive yield losses. The filtration device needs to be balanced towards the application. It should, therefore, be tested accordingly.

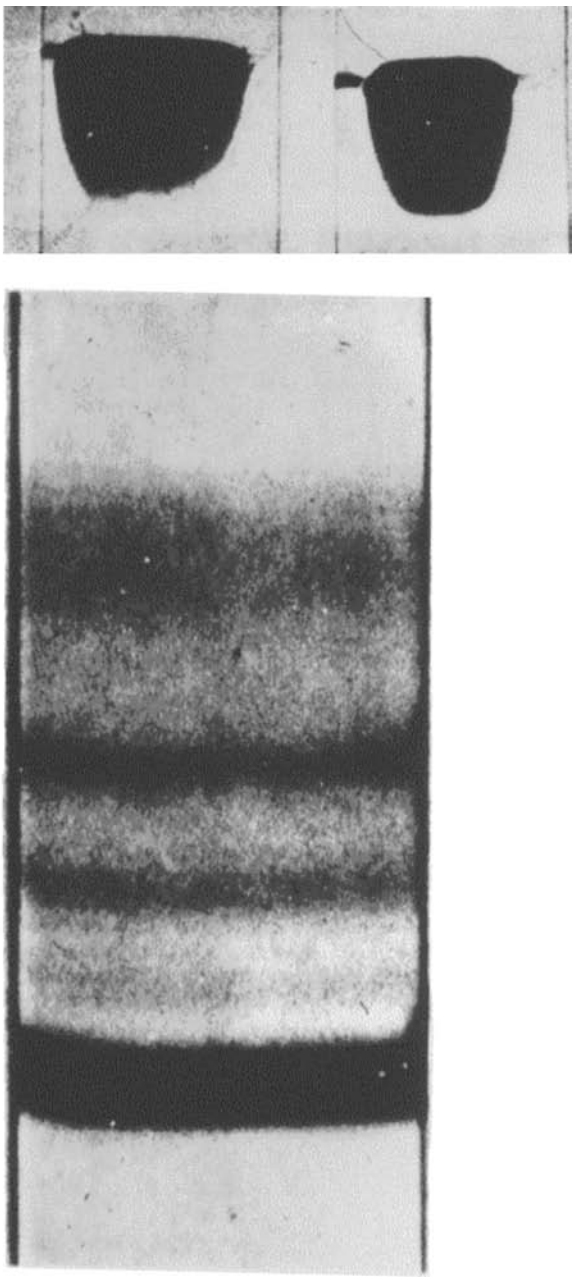


FIGURE 9 (**A**) Electrophoresis: serum on cellulose nitrate. (**B**) Electrophoresis: serum on cellulose acetate. *Source*: From Gebott et al., 1969.

PROTEIN-FILTER ADSORPTIONS

The study of protein adsorption to filters makes use of "the filter saturation curve." What is measured is the concentration of the filtrate as a function of the volume filtered per unit area of filter. Initially the rate of adsorption is highest, while the filtrate's protein concentration is the lowest. As the filter approaches saturation, the adsorption rate decreases. The effluent's protein content increases until it approaches that of the

feedstream. At this point, by definition, no additional adsorption can occur. The data reveal the volume of a given protein solution needed to saturate the particular filter. In addition, the quantity of protein required to saturate per unit area of filter, the filter's effective filtration area (EFA) Becomes known. Generally, this approximates 5 to 50 liters per 10 inch filter cartridge; which translates to 0.5-10 mL per cm^2. The concentration of the protein solution and the area of the filter being known, the protein adsorption value per unit filter area at the point of saturation can be calculated. For a solution of a given protein concentration, the volume necessary to be filtered for the effluent to achieve parity with the concentration of feedstream becomes known.

Sundaram (1998) points out that in most cases the filter saturation curve indicates that a comparatively small amount of protein serves to saturate the filter to attain the steady state of quantitative recovery in the effluent. To the extent that the adsorptive propensities of the different filter types are investigated to learn which adsorb least, the effort is largely unnecessary because so little protein is lost regardless. The attendant economic concerns would still hold for filling containers with very dilute protein contents, or with very expensive protein, or where small batch sizes are involved. Sundaram further calls attention to the potential loss of protein by adsorption to the surfaces of the processing equipment other than the filters. Prefilters, tanks, rubber or metal piping, even filter holders, etc. need to be considered. To avoid this overall adsorptive loss, careful system design is required.

Undissolved Protein

There are instances where at steady state the effluent's protein concentration does not recover fully to that of the feed solution. The phenomenon of not achieving "saturation" may result from the sieve removal of proteinaceous gels or particulates, or from undissolved protein. Such items of high protein content credit exaggerated initial protein concentrations to the solution. Protein deposited during filtrations using microporous membrane may have been generated as small quantities of aggregated or denatured protein caused by foaming; extreme pHs; high shear stresses; or extreme temperatures (Sundaram, 1998). This is more likely to occur the finer the pore size rating of the filter, as would be used in organism or virus removal. The condition may also stem from multilayered adsorption wherein the saturating mono-layer of protein participates in protein-protein interactions to build in depth.

The latter possibility is disagreed with, especially by those who define "adsorption" by way of the Langmuir adsorption isotherm wherein only one layer of adsorbed material is considered possible. The disagreement may reflect differences in semantics. Some hold that the term "adsorption" pertains only to the partitioning of a macrosolute between a solvent and a surface; thus defining the adsorbed material as being that limited to intimate contact with the adsorbing surface. Be that as it may, in the forthcoming discussion of the electric double layer it will be seen that while the adsorptive forces do indeed hold the primary adsorbed layer fixedly, a more distant layer is also held, albeit not as fixedly. The point being made is that in actuality the adsorptive forces extend further than just to the first layer, although in less strength. The differentiation among adsorptive effects by way of definitions may satisfy attempts at classifications. A more meaningful differentiation will result from characterizations based on the effects that are manifested by the mechanisms of adsorption.

To make certain that protein in particle form is not classified as being protein adsorbed from solution, filters of smaller pore size ratings may be employed to remove such non-dissolved matter. Adsorption studies based on this usage are obliged to assume

that the extent of pore wall surface is the same for the filters of different pore size ratings. Such an assumption may be unwarranted. The numbers used in listing filter porosity values are likely approximations. This tends to render as unreliable the adsorption data based on unit surface area studies.

A similar consideration likely extends to the sometimes advocated substitution of 0.1-μm-rated membranes for the 0.2-/0.22-μm-rated filters. Quite probably the finer pores of the 0.1-μm-rated filter of the same porosity present larger pore-wall surface areas than do their 0.2-/0.22-μm-rated counterparts.

Dynamic or Static Wetting-Out

Pitt (1987) in his investigative work brought the filter into contact with the protein solution by dipping. Utilized were filter disks of 8 mm diameter having 0.5 cm^2 nominal area, dipped in 1 mL of protein solution for 18 h at ambient temperature. The protein, partly labeled by ^{125}iodine, was used with filters of hydrophilic PVDF, hydrophilic polysulfone, and cellulose diacetate. These filters showed low protein binding on the order of $< 10 \mu g/cm^2$ nominal area for bovine serum albumin (BSA), and IgG from sheep. This is what would be expected from hydrophilic surfaces comparatively incapable of hydrophobic adsorptions. Polyamide (nylon), and cellulose nitrate filters exhibited high protein binding for these proteins, namely, ~190 and ~250 $\mu g/cm^2$ nominal area respectively. These findings could also be expected from the adsorptive mechanism based on hydrogen bonding, especially in the case of the nitrocellulose polymer.

Long experience with the relative difficulty of wetting filters demonstrates the inefficiency of this static method. It may prove difficult to expel the air from the smaller filter pores. What is required is the dynamic flow of the protein solution through the filter. The complete involvement of all the pore surfaces should not be assumed. It may necessitate prolongation or repetition.

Pitt (1986) compared the static method of dipping with the active or dynamic method of passing the protein solution through the filter's pores by filtration. Only the nylon (polyamide) filter among a group of three, including hydrophilic polysulfone, and hydrophilic PVDF, showed a difference in the amount of protein adsorbed in terms of the mass per unit area uptake of insulin or of human growth hormone. The nylon filter adsorbed a much higher amount of protein by using by the dynamic rather than the static method; namely, $> 1000 \mu g/cm^2$ nominal area. Certain nylon filters are reputed to be somewhat "open." Perhaps, it may be speculated that such a "more open" filter responds more strongly to the greater efficiency of the dynamic method of exposure to the protein solution.

Figure 10 illustrates that filters made of the same polymer and having the same pore size rating, but made by different manufacturers exhibit different adsorption characteristics. The variations may well result differences in the pore size rating systems. There is no industry wide pore size rating system.

The Electrical Double Layer

The situation being considered is that of two surfaces, both immersed in an aqueous solution. One may be of a particle or large molecule; the other that of a filter. Common to all surfaces, the surface molecules differ from those within the mass because they interface with a surrounding phase that contains relatively few molecules that are similar enough to bond with. The surface layer is, thus, molecularly unsaturated. This gives rise to a residual field that finds expression in a surface energy. As is inevitable, the surfaces,

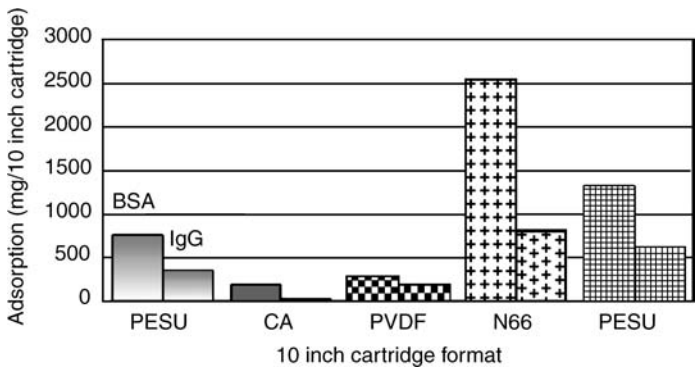


FIGURE 10 Different proteins on given polymers and given proteins on different polymers. *Source*: From Brose and Weibel, 1996.

because of their higher energy, acquire ions from the solution, or gain charges induced thereon. The acquired ions become fixed to the immersed surfaces.

In effect, they simultaneously endow the surfaces of both filter and protein, (or other entity), with electrical forces of both stronger and weaker powers, and of both attractive and repulsive capabilities. The magnitude of these effects is in accord with the charge density on the individual adsorbing surface resulting from the fixed charges. The acquired ions give rise to the strong and, hence, long range coulombic forces. When the surfaces of both organism and filter bear charges of like sign, their mutual repulsion ensues. The attractive forces of opposite signs are effective only at shorter ranges. Like the VDW forces, they cannot redress the repulsion. The limitation to the effectivity of the VDW forces to short distances is a measure of their weaker influences. They avail only at shorter distances.

As would be expected, the electric potential of the surface charges just described is moderated by counterions from within the solution that become fixedly attached to them. These intervening counterions effectively shield the electrical interactions between the charged surfaces. In consequence, the electrostatic forces are reduced in potential.

Not all of the surface charges are neutralized or shielded by the inseparably-attached counterions. Therefore, more ions of opposite charge, intermingled with their counterions, are attracted by the fixed surface-charges that remain unshielded. However, these counterions remain mobile because they are increasingly distant from the fixed surface charges, although responsive to them. The interactive forces diminish sharply with distance. Other than the first layer of counterions that becomes firmly attached, the remainder of the counterions involved, although attracted, are sufficiently distant from the surface charge to remain mobile within the solution. They can be caused to migrate by the impress of an electric current. This establishes a line of separability between the fixedly attached counterions and those that are mobile.

The electric potential that from this line of counterion separation extends further into the liquid to satisfy the un-neutralized portion of the surface-fixed charges is called the zeta potential. The distance it extends is known as the Debye length. In effect, the zeta potential measures the unsatisfied electric charge of the single layer comprised of the ions of the surface and the somewhat fewer counterions they fixedly attracted and bonded. The layer of fixed surface charges and their tightly bound counterions is the first of the double layers. The mobile counterions in the solution constitute the second of the double layers. In combination they form the electric double layer.

It should be remembered that the electric potential characterizes both the filter and particle or protein surfaces: The larger the Debye length, the greater the electric potential,

and the more extensive the distance between both surfaces. Although attractive like-charges and repulsive opposite-charges are simultaneously active, the stronger repelling like-charges dominate at the longer Debye lengths. The repulsion derives from the influences of the strong primary forces that are operative over longer distances. The other repulsive force is more general. It results when any two atoms are brought together close enough for their (negatively charged) electron clouds to overlap.

For the attractive influences to exercise their powers the repulsive forces require attenuation. This situation is invoked by the addition of salts to the solution. As the ions thus supplied increase in concentration, the counterion cloud around each surface increases as well. The enveloping ions shield the zeta potential, and the Debye length decreases proportionally. This enables the weaker attractive forces to assert their powers. The attractive forces are short-range and electrostatic. They are the products of various dipole interactions, including VDW forces. The result is an adsorptive joining of the two surfaces, namely, that of the protein or particle and that of the filter.

Exactly the same phenomenon applies to the different particles constituting a colloid. The colloidal particles separated by the zeta potential are enabled to agglomerate and precipitate when salted out, as by the addition of the multi-ions of aluminum sulfate in water clarification contexts.

Incidentally, the equilibrium point where the attractive and repulsive forces balance one another defines the space that exists between the atoms composing molecules, and also between the overlapping segments of long polymeric chains.

When placed in an electric field, dipoles will tend to orient themselves in head-to-tail chain-like fashion alternating their positive and negative partial-charge interactions. Such an array of dipoles intervening between two like-charged repulsive forces serves to attenuate their mutual antagonism by substituting a chain of subsidiary or partial-charge interactions. As a result of the diminishment of the repulsive forces, the countervailing forces of attraction assert themselves, and the two surfaces become united by the adsorptive sequestration mechanism.

Dipole Alignment

The zeta potential and its consequent extensive Debye length are reduced by high ionic strengths such as are occasioned by the hydronium ions of low pHs, or by the ions of soluble salts. Upon the reduction of the Debye length as occasioned by the addition of ions, the short range attractive VDW forces enable the appropriate surface sites on the organism to interact with those on the filter. Since there are as many opportunities for the VDW force orientations to exercise repulsions, as there are to encourage attractions, the domination by the attractive tendencies requires explanation. It turns out that two factors incline towards the attractive forces. The induced dipoles that are the VDW forces in a molecule can align themselves either in a head-to-head and tail-to-tail, or head-to-tail sequence. This reflects a probability factor. The head-to-tail arrangement involves a lower energy level. This orientation is, therefore, favored.

This is abetted by a polarization factor. Also called the London dispersion force, it affects the size of the dipole moment by influencing the spatial separation of the plus and minus charges is each molecule in proportion to the dipole sizes. In the electrostatic attraction mode, polarization increases the interaction between the two molecules. In the repulsive orientation, the polarization decreases the molecular moments, and, hence, the repulsive force. As stated by Wheland (1947), “The attractive orientations on the average, are more attractive than the repulsive orientations are repulsive.” As a result of these two

factors, the short range VDW forces exert an attractive influence, and bring about the desired filtrative removal of organisms by an adsorptive interaction.

This phenomenon can also be stated slightly differently. When placed in an electric field, dipoles will tend to orient themselves in head-to-tail chain-like fashion alternating their positive and negative partial-charge interactions. Such an array of dipoles intervening between two like-charged repulsive forces serves to attenuate their mutual antagonism by substituting a chain of subsidiary or partial-charge interactions. As a result of the diminishment of the repulsive forces, the countervailing forces of attraction assert themselves, and the two surfaces become united by the adsorptive sequestration mechanism. This is perhaps best illustrated by the colloidal destabilization phenomenon by way of which study it was elucidated. It is also this effect that minimizes the strength of the (repelling) zeta potential.

Debye Length Phenomena

Fletcher (1996) describes the attractive and repulsive forces involved in these interactions between organism and the filter or other surfaces, and offers quantification of the approximate distances at which they are significant: At distances greater than 50 nm, called the "secondary minimum," VDW attractive forces do cause the positioning of the bacteria nearer the surface; but so weakly that they are readily removed by shear forces. At distances of 10–20 nm, repulsive electrostatic forces dominate. Here the Debye length is too great for the weaker VDW attractive forces to participate. At between 2 and 10 nm both repulsive and electrostatic attractive forces are manifest, the distance now being small enough to allow the weaker attractive forces to begin asserting themselves. At between 0.5 and 2 nm, the water adhering to the surfaces is still a barrier to specific surface interactions. However, if the two surfaces include non-polar areas or patches, these may coalesce to form hydrophobic adsorptions concomitant with the elimination of the interfering water. At less than 1.0 nm, the attractive forces are strong enough to cause specific organism/ surface interactions to take place.

Pall et al. (1980) state that when the Debye length corresponds to a zeta potential of less than 30 mV, the VDW forces can enable the adsorptive interaction. Colloids are destabilized when their particles are endowed with net surface charges of similar sign in the magnitude of 30–40 mV or more.

There are, however, limitations to attaining the required small Debye lengths. The adsorption of a surfactant molecule, or of a macromolecule, onto a surface increases its size. This steric hindrance may keep it too far from the other surface to yield a short Debye length. Non-ionic surfactants in particular contribute to such a condition. The two surfaces are, thus, sterically stabilized against adsorptive coalescence. Spatial interferences can also result from the hydration barriers created by the increase in an ion's size by its acquisition of waters of hydration. Such steric or geometric interferences can create an insurmountable energy barrier sufficient to frustrate adsorptions.

Recent Cartridge Testing

A plentiful literature is available on protein adsorption and its effects. Its accumulation over time spans periods when factors presently considered important were then not recognized to be so. Thus, the value of some earlier studies can be questioned. Additionally, much exploratory work dealt with filters in small disk form. While often appropriate for experimentation, they obviously do not suit processing needs. Consequently, the forthcoming review of recent work will be confined to investigations

involving filters in cartridge form. Conclusions drawn from such studies should be made with care. Investigations made using only membranes and proteins are designed to evaluate the adsorptive tendencies of the constituting polymers. This could enable filter choices to be made based on the polymer's character with regard to adsorptions. The results obtained using small disk filters can be extended to measure the contributions of the filter holders, and of ancillary equipment exposed to the protein test solutions. At best, only the polymeric interactions are assayed.

However, the uncomplicated simplicity of the small filter disk may not translate sufficiently well for cartridge usage in processing operations. The more complex cartridge constructions involve pleating of the filter; the pleat densities, and their heights, and patterns; along with the nature of the separation and drainage layers that are used; along with its endcaps, and core and cage of whatever materials of construction deserve consideration. These several items all contribute to the totality of the cartridge's adsorptions.

The performance of a cartridge regarding its protein adsorption is at the most a characterization of cartridges of its type. Its performance may be compared for whatever reason and by any standard, however arbitrary, with cartridges of a different type. However, in making comparisons there should be an awareness that the construction features of the tested cartridges are unalike. Tests reported in the literature distinguish the subject cartridges according to the filter's polymeric identity. They are further identified as being hydrophilized or not. It should be remembered that if the surface's conversion will totally insulate the underlying polymeric membrane from contact with the protein. Care must be taken not to misidentify the polymeric material that is in actual contact with the protein solution.

Filter Saturation Curve

The following statement, previously made, is here repeated for convenience in referencing:

> The study of protein adsorption to filters makes use of "the filter saturation curve." What is measured is the concentration of the filtrate as a function of the volume filtered per unit area of filter. Initially, when the protein solution encounters the filter, the rate of adsorption is highest, while the filtrate's protein concentration is the lowest. As the filter approaches saturation, the adsorption rate decreases. The effluent's protein content increases until it approaches that of the feedstream. At this point, by definition, no additional adsorption can occur. The filter's adsorptive power is saturated. The data reveal the volume of the given protein solution that was needed to saturate the area of the particular filter.
>
> Plotting protein recovery (in percentage) against volume throughput (in liters) will show a straight horizontal line when the filtrate's protein concentration attains the steady state equal to that of the feed stream. A continuation of the line's upward slope to the end of the test signifies that the filter's adsorptive capacity had not yet been assuaged to that point.
>
> A straight horizontal line from the onset to the end of the filtration test signals that there is no adsorption, or so little that the rate and/or extent of adsorption is not noticeable enough to affect its effluent concentration over the course of the filtration. The likely interpretation would be that only a miniscule amount of adsorption took place over a very short-time period.

An initial downward direction of the plotted line indicates the rate of protein adsorption by the magnitude of its angle from the horizontal. A subsequent upturn of the

plotted trace signals by its steepness the diminishing rate of protein uptake by the filter. Its eventual return to the horizontal bespeaks the steady state of saturation.

Where protein adsorption is the concern, that filter would be considered best that indicated from the start the least deviation from a straight horizontal line.

The filter saturation data can be plotted in the manner of the classical Langmuir adsorption isotherm as protein adsorption (mass/unit area) versus the bulk protein concentration (mass or volume). As the rate of adsorption decreases, the line tends to level on its way to the horizontal. It does so at the point of saturation. The failure of the plotted line to achieve a horizontal position means that the protein uptake never reached saturation over the duration of the test. The area under the curve is a measure of the amount of protein adsorbed over the test's duration. In the case of cartridges this includes the amount of protein adsorbed by all components and appurtenances in contact with the protein test solution. A plot of protein adsorption as a function of time reveals the kinetics involved.

STUDIES DIRECTED TO CAPSULES

Protein adsorption studies involving cartridges and disposable capsules suitable for processing purposes have been reported in the literature. The data garnered in such experimentation will here be reported by way of the graphs or tables constructed by their investigators. Except where necessary to explain our conclusions, experimental details will not be dealt with. Interested parties can secure the experimental details from the published papers. This will enable readers to make their own evaluations.

Brose and Waibel (1996) studied the uptake of BSA, and of IgG using commercially available filter capsules constructed with a polypropylene shell. The capsule designs differed to a degree: the PVDF capsule did not utilize a prefilter; while the CA device included a 0.35-μm- rated CA prefilter along with a 0.2-/0.22-μm-rated CA final filter.

These filter units consisted of membranes of either PVDF, nylon, or CA of 0.2-/0.22-μm-rated pore size. Dynamic flow-through exposed the membranes to the protein solutions at 24°C under < 1 psi over a time period that usually terminated when protein adsorption/desorption attained equilibrium. Figure 11 includes a plot for the CA membrane. The concentration of adsorbed BSA in (wt %) is plotted against the filtrate mass (grams). The protein feed concentration was 5000 μg/mL (0.5 wt %) in phosphate buffered saline at pH 7.4. Figure 12 shows that the BSA ($\mu g/cm^2$) adsorption data plotted

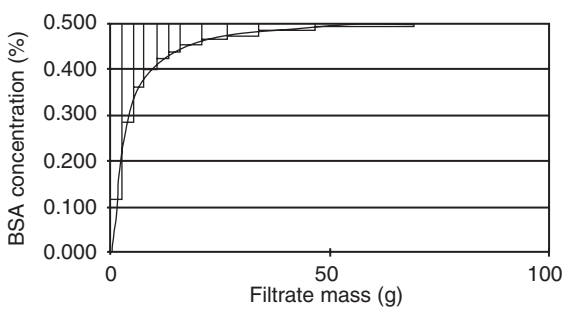


FIGURE 11 Adsorption of BSA versus Filtrate volume. *Source*: From Brose and Weibel, 1996.

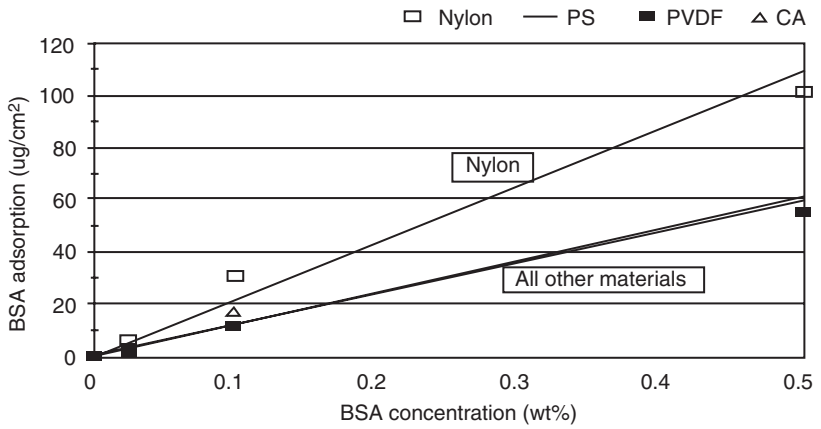


FIGURE 12 Adsorption versus protein concentration (BSA). *Source*: From Brose and Weibel, 1996.

against the BSA concentration (wt %) was linear for all three capsule types. However, the BSA adsorption, at about 110 μg/cm^2 by the nylon (polyamide) filter device was almost double the capacity of either the CA or PVDF filters. These equaled one another at about 110 μg/cm^2. The substantially greater adsorption of both BSA and IgG by nylon in capsule form is in line with findings by Pitt (1987). His earlier work was based on the testing of nylon filters in the form of single pieces of membranes unencumbered by considerations of holders, etc. With regard to IgG, the nylon capsule did not correspond in linear fashion at low levels. It did attain the saturation stage of the adsorb/desorb equilibrium at a high adsorption level.

Figure 5 indicates that IgG adsorption (μg/cm^2) is linearly related to its concentration (wt %) for the CA and the PVDF membranes; the level adsorbed being ~200 μg/ cm^2/wt %. The nylon filter adsorbed each protein in a non-linear fashion until saturated with it. Brose and Waibel attribute the high protein adsorption of the nylon to the strong hydrogen bonding of the amide groups of the 6,6 nylon polymer.

In each case the nylon showed itself to be the most adsorptive of the tested filters for both the BSA and IgG proteins. This agrees with Pitt (1987) findings. BSA demonstrates an adsorption relationship that is linear with respect to its concentration for all the tested capsules. However, at 220 μg/ cm^2 wt%, it is almost double the 120 μg/ cm^2 value exhibited for the cellulose ester and PVDF instruments. In the case of the IgG adsorption, it too is linear relative, at 200 μg/ cm^2, to its concentration for the CA and PVDF capsules. For the polyamide (nylon) device, the adsorption increased linearly from its starting level to the point of saturation.

It is evident that protein adsorption by filters occurs, that it is manifested differently by different proteins and filters, and that its control, where desired, is variously managed in separate ways that do not find universal application to all proteins or to every filter.

STUDIES DIRECTED TO CARTRIDGES

The performance of various polymer type membranes in 10-inch cartridge form in the retention of protein was investigated by Datar et al. (1992). The use of cartridge filters

instead of flat membrane disks (13 or 47 mm) enabled the protein retention influence of the conventional support and drainage layer construction to be assessed. The effect of prefilters could also be measured as four different types were evaluated along with four final filters. Certain prefilter-final filter combinations were also tested. The protein used was BSA in 90μg/mL concentration. Protein concentration was measured as adsorption units at wavelength 280 nm and converted to concentration units as μg/mL.

The filter types examined were:

1. polypropylene membrane rated 1.2 μm
2. polypropylene depth filter rated 0.5 μm
3. nylon 6,6 membrane rated 0.2 μm
4. hydroxyl-modified polyamide membrane rated 0.2 μm
5. hydrophilic PVDF rated 0.2 μm
6. mixed cellulose ester depth filter (not rated)
7. CA membrane rated 0.2 μm
8. polysulfone membrane rated 0.2 μm

The protein saturation curves depict the pattern of protein adsorption forthcoming from the tested cartridges. The CA unit, as well as the hydroxylated polyamide, and the hydrophilic PVDF were essentially equal in their small uptake and their swift saturation with the BSA. This is indicated respectively by Figure 7A and 7B. The results are in agreement with the known experience wherein hydrophilic membranes are found not to favor protein adsorptions.

The membrane identified as being constructed of polysulfone polymer shows, within 1 or 2 L of flow-through, so thorough a recovery as to have been extraordinarily fast in becoming saturated with whatever small quantity of BSA it adsorbed. Polysulfone is sufficiently hydrophobic, however, to have been expected to be more avid in its hydrophobic adsorptions. Albeit real, the behavior may simply be inexplicable for now. It is also possible that additives, such as wetting agents, are part of its formulation, and are responsible for its unexpected behavior. Possibly being considered subsidiary, they may not been mentioned; even were they were known to the experimenters.

Notice should be taken of the BSA adsorption by the nylon 66. It was strong from the very start, recovered its concentration somewhat slowly, and achieved its saturation more gradually than did the other polymeric cartridges. Of the cartridges tested nylon was the most adsorptive of BSA. This conclusion affirms the common view regarding nylon 6,6 as being strongly adsorbing of proteins.

The prefilters tested as follows: Despite its innate hydrophobicity, polypropylene in both pleated membrane and depth filter form caused significant protein loss. Both cartridge types attained maximum concentration recovery after a flow-through of 1 or 2 l.

The cartridge of mixed cellulose esters initially adsorbed high amounts of protein. Its recovery to the protein concentration of the test solution was the most gradual of all the cartridges tested. Although identified by way of its manufacturer and catalogue title, its polymeric composition was not specified. The mixed esters are a mixture of CA and cellulose nitrate. The adsorptive take-up of protein by CA is generally low. That of the cellulose nitrate is very high, due to the hydrogen bonding by the nitro group of atoms. The slow recovery shown by this cartridge may be explained as resulting from two different polymers, each distinctive in its own rate of achieving saturation. Had the description of the cartridge's polymeric identity been made plain, the results of the protein adsorption and its recovery would have been evident. This argues for a fuller characterization of the filters used in investigations to include their polymeric identity, and that of additives. This could assist reaching proper experimental conclusions.

Having tested both prefilters and final filters, Datar et al. (1992) assayed various combinations of both in an effort to construct a filter system least conducive to protein adsorption. The nylon prefilter (single layer)/nylon final filter (double layer) combination; as also the hydroxyl-modified polyamide prefilter (single layer)/final filter of the same material in a double layer were examined. In essence each set of filters exhibited, with only slight differences, if any, the protein adsorption characteristics shown by the final filter alone. As might have been expected, the prefilter/final filter combination composed of membranes hydrophilized to present hydrophilic surfaces were the least involved in protein (BSA) adsorption.

SUMMARY

What was sought in this exercise was an understanding of the filter cartridge properties important to the adsorptive retention of proteins. The literature pertinent to the subject is impressive in both quantity and quality. Our selection and treatment of it was directed primarily to the polymeric materials comprising the filters in terms of their tendency towards adsorptions in general, and towards proteins in particular. Comparisons among filters on this basis are plentiful, but the techniques applied vary in their degrees of sophistication. Anticipating an address to production needs, our focus was extended to the modification, if any, of the polymers contribution by the features of cartridge construction. On this point, the literature is sparse. Details regarding additives to the polymeric compositions were lacking.

Our conclusions confirmed that hydrophobic areas within a heterogeneous surface were promotive of protein adsorption. The hydrophilic, charge-involving areas of filter surfaces were significantly less encouraging to protein uptake. To the minor extent examined, neither membrane pleating nor the cartridges' drainage and separation layers exerted a notable influence on the adsorption of proteins. In our judgment, the prefilter contribution to protein adsorption did not modify, in proportion to its added area, that of the final filter.

Much remains to be systematically investigated. In designing a filtration system that would minimize protein adsorption, an optimum ratio of prefilter to final filter would require experimental definition. The extent of filter area to be used in a production run would necessitate an experimentally defined EFA. Where the minimum adsorptive losses would be desired, the smallest EFA should be used. This would be of particular concern where high-value protein would be at stake, or where dilute protein solutions undergo filtrations, as for organism or viral sterilizations.

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11
The Filter Integrity Tests

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BACKGROUND

The integrity testing of filters is one of a series of interdependent activities that in their proper combination result in the preparation of sterile drugs. Integrity testing, bioburden studies and process validation are the building blocks of this practice. Each of these components has its own complexities, and not all of their influencing factors are presently understood.

The integrity testing of filters is central to the practice of sterile filtration. The exercise is seen to stand between certainty and potential failure. Because of their frequent usage the integrity tests that are available are generally well known. What is less well comprehended is their underlying physics, their operational requirements, and a deeper understanding of when a given test is particularly suited for a given application.

The key requirement of an integrity test is that it establishes a quantitative correlation between a property or characteristic of the filter of interest with its organism retention capabilities. This would, for example, address the needs of sterilizing filtration practitioners to identify filters of a particular pore size rating having the necessary bacterial retention capability.

Present integrity test procedures enable this to be done by way of interpreting measured airflows through water- or solvent-wet membranes. The purpose of this writing is to help clarify the situation.

NON-DESTRUCTIVE TESTING

An important application of the filtration process is the production of sterile drugs. A method of identifying a "sterilizing grade" filter, one suitable for *trial* in a sterilizing filtration, is made by way of integrity testing. The FDA has defined a sterilizing filter as one that retains the classic challenge of at least 1×10^7CFU of *Brevundimonas diminuta* ATCC-19146 per cm^2 effective filtration area (EFA) at pressures up to 30 psi (2 bar). It

should be understood, however, that a filter thus qualified does not automatically ensure a sterile effluent. That achievement is the result of several factors whose successful culmination requires the documented experimental evidence that constitutes validation. In the performance of the sterilization exercise it is necessary to ensure that a proper filter, a "sterilizing grade" filter, is being utilized, one capable of retaining the FDA-defined organism challenge[a] This is best assessed by a direct confrontation of the membrane by a proper organism challenge. However the filter, thus tested, is contaminated by the organisms and may not subsequently be employed as a process filter. What is required is a non-destructive integrity test, based on correlation of pore size with the log reduction value (LRV) indicative of organism retention. Therefore, a surrogate test is used that is non-destructive to filters, and whose values will correlate with organism retention.

In the present practice the membrane's largest pore size is probed by the bubble point test. Its value can be correlated with the membrane's ability to sustain the 1×10^7/cm^2 *B. diminuta* confrontation made by the use of a standard ASTM 838-05 microbiological challenge procedure. On this basis such membranes are experimentally qualified to be of the "sterilizing grade," and are thus labeled as being of the 0.2-/0.22-μm-rated pore size. They may then be selected for trials in actual product filtration sterilizations to ascertain their suitability to so perform in given filtration contexts.

Filter manufacturers have measured and correlated bubble point/diffusive flow values and LRVs for the commercially available membranes. It is this correlation that serves as a non-destructive test for determining a given filter's organism retention capabilities. This is precisely the information needed to ensure the proper choice of membrane for use in filtrative sterilizations.

REGULATIONS

So important is integrity testing that it is required by the regulating authorities responsible for overseeing pharmaceutical processing practices worldwide. For examples,

1. FDA Guidelines on Sterile Drug Products Produced by Aseptic Processing (FDA, 2004): "Normally, integrity testing of the filter is performed prior to processing, after the filter apparatus has already been assembled and sterilized. It is important that integrity testing be conducted after filtration to detect any filter leaks or perforations that might have occurred during the filtration. Forward flow and bubble point tests, when appropriately employed, are two integrity tests that can be used. A production filter's integrity test specification should be consistent with data generated during filtration efficacy studies."
2. Guide to Good Pharmaceutical Manufacturing Practice (Orange Guide, UK, 1983), p. 62 9.82: The integrity of the filter assembly should be checked by an appropriate method, such as bubble point pressure test or forward-flow pressure test immediately

[a] The stipulated challenge of 1×10^7 of *B. diminuta* per cm^2 was proposed by HIMA (1982) for flat disc membranes, to be achieved at 30 psi (2 bar). *The total effluent was to be assayed*. In the case of cartridges, the effluent volume required being limited, for practical purposes, to that quantity produced at rates of flow of 3.86 l/min per 0.1 m^2 EFA. A cartridge containing 7 feet^2 (6503 cm^2) of EFA would require that a flow of 5.4 l/min be used in a challenge to define a "sterilizing grade" filter.

before and after use. Abnormal filtration flow-rates should be noted and investigated. Results of these filter integrity checks should be recorded in the batch record.

3. ISO/DIS 13480 2, 2003-03-15, 'Aseptic Processing of Health Care Products – Part 2: Filtration' Section 7: Filtration Process

> 7. 1.2 — Written integrity test procedures shall be established including acceptance criteria and methods of failure investigation and conditions under which the filter integrity test can be repeated.
>
> Notes:
> —Information from the filter manufacturer can be useful in designing and validating integrity test procedure(s) based on gas flows through wetted filters.
> —It should be demonstrated that the integrity test conditions can be supported by standardized bacterial retention testing. The standard bacterial retention tests should use a challenge level of at least 10^7CFU/cm^2, with filters representative of standard production filters approaching the acceptance test limit.
> 7.1.3. One or more wetting fluids shall be selected. These shall be the filter manufacturers' recommended reference wetting fluid or the actual fluid to be filtered. In the latter case, the appropriate integrity test value specification shall be established and validated.
> 7.1.4. For air and gas filters, appropriate frequency for physical integrity testing shall be established.

Practical Concerns Apropos of Regulations

The regulators in fulfilling their obligation to protect the public may establish rules likely to reduce risk to a minimum. Such may, however, encounter practical limitations.

For example, ISO/DIS 13408, Section 8.10 states: "The filtration system should be designed to permit in-place integrity testing as closed system prior filtration." However, Wallhaüsser (1985) believed that the diffusion test would be impractical as a pre-use integrity test were steaming used as the sanitizing agent. Given the sensitivity of diffusive airflows to temperature, the testing would require being delayed until the system, filter and housing, cooled to the level proper for measurement; a matter of many hours. Scheer et al. (1993) explored the effects of temperature on the diffusion test; it is the least subjective of the integrity tests. These investigators found that serious errors in test results are possible unless temperature and volume factors are recognized and accommodated. They observed, "The exigencies of field filter testing may only rarely allow the needed degree of control." Similar caution has to be taken with bubble point measurements as the bubble point depends on the surface tension of the wetting fluid, which respectively depends on its temperature. When integrity tests are performed the temperature of wetting fluids, test gas and the environment need to be diligently observed as temperature changes have a detrimental influence.

The alternative to a substantial time delay would be to integrity test the filter under aseptic conditions outside its holder, and to install it under the same conditions. This would eliminate the testing delay occasioned by the need for steam sterilized filters to cool to where they could be used for accurate airflow measurements. Several filters could simultaneously be sanitized outside the filtration system and be allowed to cool in an aseptic ambiance until one was needed for installation.

To comply with recommendations the filter would be sanitized after its installation, but prior to its being integrity tested in-place. Ironically, manual testing of the already installed filter would be accomplished by downstream measurement of the airflow rate.

Such would risk asepsis. However, upstream integrity testing can be performed with automated integrity test equipment.

When to Perform Integrity Testing

Another example of regulations, at times, being in opposition to practicality is given in answering an often asked question, namely, when integrity testing should be performed. Most often the regulations require the filter's integrity testing to be conducted "immediately before and after use." However, regulatory guidance regarding pre- and post-use integrity testing is a bit ambiguous. For example, FDA's aseptic processing guidance (2004) states, "Integrity testing of the filter(s) can be performed prior to processing, and should be routinely performed post-use." Annex 1 of the European GMP regulations states, "The integrity of the sterilised filter should be verified before use and should be confirmed immediately after use by an appropriate method such as a bubble point, diffusive flow or pressure hold test." Cleary regulators expect a post-use integrity test to be performed, while a pre-use integrity test appears to be optional, at least in the United States.

Irrespective of the regulatory guidance, pre- and post-use integrity testing is desired to demonstrate the integrity of the filter and to ensure the filter device has not been damaged, for example, during steam sterilization or even transportation. Ideally, the pre-use integrity test should be performed by the filter user with the filter installed in the housing of intended use; however, it is possible under certain conditions to accept the filter manufacturer's integrity test value as the pre-use test. This can be done if the individual filter has been integrity tested by the manufacturer (and is accompanied by certification to that effect) and the installation and sterilization processes have been validated, and the personnel appropriately trained by the filter user to assure, insofar as possible, that these operations are well controlled and do not result in seal leakage or damage to the filter. In the event of a post-use integrity test failure, the filtered batch must be rejected.

Pre-Use Integrity Testing

After sterilization of the filter cartridge, for example, by steam, it is recommended (FDA, 2004) that it be integrity tested prior to filtration. (Post-filtration integrity testing is a requirement.) There are good reasons for this recommendation. Stresses, possibly induced to the filter during its manufacture, are relaxed by the steam heat. This may result in altering the sizes of some pores.

The initial integrity testing may be performed as soon as the membrane is thoroughly wet. Integrity testing can be performed off-site prior to the filter's installation in its housing. However, aseptic installation is then necessitated. This involves risk. Integrity testing after installation introduces its own problems. Manual testing compels invasion of the system downstream of the filter. Serious risks to the system's asepsis are involved. The use of automated test machines avoids this problem. They all utilize test methodologies which operate from the upstream side and, therefore, avoid such compromises. The available automated machines utilize either pressure decay, or direct flow measurements, making it unnecessary to attach anything to the downstream side.

Since the steam sterilization process is commonly the most stressful process experienced by the filter, it would be advantageous to test the filter after steam sterilization but before use. Any potential damage resulting from excessive sterilization parameters or incorrect handling would be determined by such test. However, since the filter requires wetting and testing with an atmospheric pressure condition on the filtrate

side, the filtrate side at that point requires appropriate engineering designs to allow the flush, drain, and test process. These designs are not easily fulfilled, and the risk decision should lie within the hands of the drug manufacturer using risk management perspectives.

Post-Use Integrity Testing

Other than convenience, there is no reason to delay the post-use integrity testing. There is not a sufficient experience on which to formally base a time limit on the commencing of the test. Nevertheless, a delayed testing can only encourage time-related alterations, if any, in the filter's condition; nor need any be elucidated. What is wanted is the knowledge of the post-use integrity of the filter. Delaying the analysis can only occasion change, from whatever cause, which can modify the filter's immediate after-filtration condition from what it was.

Integrity Test Purposes

Integrity testing serves several purposes. One important function is to make certain the identity of the selected filter; by affirming the correctness of its identifying label. Each pore size rating of a filter is characterized by the filter manufacturer as having a distinct integrity test value. At the moment when a filter is removed from its shipping container preparatory to use, only the proper performance of an integrity test attests to its identity. Even its identifying label is no guarantee; mistakes do occur. Integrity testing provides the needed confirmation that the selected filter is the proper one.

The filter and the liquid vehicle being filtered may be incompatible. Such may result in pore size changes consequent to the membrane's exposure to the fluid over a time period peculiar to the specific membrane (polymer)/liquid couple (Lukaszewicz and Meltzer, 1980). Integrity testing is sensitive enough to disclose whether a filter has undergone even subtle incompatibilities that can impair its retentivity.

It is not uncommon to steam-sterilize a filter prior to its use. As stated, the heat may release stresses, if any, built into the filter during its fabrication. Alterations in the pore structure may result. Moreover, the steaming cycle itself with its heating and cooling phases is a stressful experience for any filter given the different rates of expansions and contractions of its several components. It may induce stresses in the membrane. Repeated steaming may lead to anomalous results. A series of repeated steam sterilization cycles, each followed by integrity testing can be used to learn how many sterilization cycles a given filter may endure. Regardless, prudence, if not economics, suggests that cartridges be confined to a single steam sterilization cycle. A comparison of pre- and after-use integrity test values can show by their constancy or lack thereof whether the membrane has undergone morphological alteration.

In the validation exercise it might be necessary to establish a relationship between the water-wetted and the product wetted membrane. This is accomplished by way of integrity testing whereby the water-wetted integrity test value is quantified to that of the product-wetted integrity test value.

Integrity testing can be performed either manually or by utilizing automated test machines. The self-evident objectivity of the automated machines offers a significant advantage. Additionally, the automated testers enable upstream measurement manipulations that finesse exposure of the filtration system to risking the system's asepsis. More important, the automated testers are magnitudes higher in the sensitivity of their measurements of airflows and displacement volumes. These greatly enhance the reliability of the bubble point's identification.

FILTRATIVE STERILIZATIONS

It is perhaps in conjunction with sterilizing filtrations that integrity testing finds a most important application. It affirms that a filter used in a sterilizing filtration is of a "sterilizing grade." And it verifies that the filter underwent no alteration in its organism-retention qualities during the entire filtration. This is done by a show of identity between the pre- and after-use integrity test values.

The concern in conducting a filtrative sterilization is that during its employment the filter may, for whatever cause, develop a pore or flaw large enough to allow the passage of organisms. One would, therefore, wish to measure the size of the set of largest pores present in the filter before and after its use to make certain that no pore enlargement occurred. Pore size determinations cannot realistically be conducted under such use conditions. But a technique for approximating the size of a filter's largest pores is available. It consists of the bubble point test. Correlation of a filter's bubble point value and its degree of retentivity is key. It makes possible the use of integrity testing to judge the appropriateness of a filter for application in filtration sterilizing contexts. The bubble point concept will presently be elaborated upon. If the bubble point value of a filter's post-use test is the same as that of its pre-use reading, the sizes of its largest pores did not undergo enlargement during the filtration. That they remained the same throughout the filtration exercise affirms the filter's integrity (Meltzer and Jornitz, 2006, Chap. 1).

Each of the differently rated pore sizes of the different filter types is distinguished by its bubble point values, and each is characterized by its particular propensity for organism retentions, namely, their LRVs. The filter's selection for the sterilization is made on the basis of its LRV. The "sterilizing grade" filter should have an LRV of 7; meaning that the filter will sustain the challenge of 1×10^7 *CFU* of *B. diminuta ATCC-19146* per sq, cm. of EFA. This accords with the FDA's definition of a "sterilizing filter" (FDA, 1980, 2004). The filter is particularized by its bubble point. Its value remaining unchanged by the filtration attests to its being of a "sterilizing grade." It has the potential to perform as a sterilizing filter under the proper operational conditions.

Similar to the bubble point, diffusive flow test limits differ with pore size ratings and polymeric molecular structures. The larger the pore size rating the lower the test pressure of a diffusive flow test. The test pressure commonly depends on the bubble point value, and is measured at around 80% of it. The membrane polymer influences both the wettability and test pressure limits. It is necessary for the filter fabricator to determine that each of its filters meets its every specification.

The "Sterilizing Grade" Filter

By definition, a sterilizing filter is one that produces a sterile effluent when confronted with the *B. diminuta* challenge (Meltzer and Jornitz, 2006, Chap. 1), just enumerated, in a standard filtration exercise based on an ASTM procedure (ASTM F 838-05) and conducted by the filter manufacturers. This does not mean that its use will necessarily produce sterile effluent in other trials. Passing the standard challenge test qualifies the filter to be of a "sterilizing grade." Its actual performance in any given application remains to be seen. Until rather recently it was believed that the sterilization of fluids could be achieved by their filtration through a "sterilizing" membrane whose proper and pertinent identity was confirmed by its pore size rating, which was itself determined by integrity testing. Developments in filtration practices showed this belief to be too generally founded. Conclusions based on pore size ratings are subject to modification by the physicochemical specificity of the organism-suspending fluid; by the individuality of

the organism type in its size-changing response to the fluid; in the possible change in pore size induced by the fluid; and by the adsorptive qualities of the filter resulting from its particular polymeric composition; all influenced by the filtration conditions in their numerous varieties, especially by the transmembrane pressure.

A filter may not sterilize the same preparation under different filtration conditions, especially under dissimilar differential pressures (Leahy and Sullivan, 1978). A given membrane may or may not retain a particular organism type suspended in a different drug vehicle (Bowman et al., 1967). The organism type need not remain constant in size, but may alter in response to its suspending fluid (Gould et al., 1993; Leo et al., 1997; Meltzer et al., 1998). The effect of the vehicle upon the polymeric membrane may cause a change in its pore sizes (Lukaszewicz and Meltzer, 1980). The complex of influences governing the outcome of an intended sterilizing filtration necessitates a careful validation of the process, including that of the filter (PDA Technical Report No. 26). The very drug preparation of interest, the exact membrane type, the precise filtration conditions, and the specific organism type(s) of concern are to be employed in the validation.

A sterilizing filter can be judged only by its performance in the removal of identifiable and culturable organisms known to be present in the drug preparation (Agalloco, 1998). Not finding any of the subject organisms of interest in a microbiological testing can be indicative of their absence, as due to their complete removal by filtration. This assumes, however, that were they present, the method used for culturing and counting them would be known, and that their proper nutrition, incubation, and adequate time for growth would have been provided for. Failing this assumption, the non- discovery of organisms does not equate with their being none.

Certainly, there is no known absolute filter, one that will retain all organisms under all conditions, especially if viruses are included (Aranha, 2004).

Log Reduction Value

Prior to use of the term LRV, now rather widely accepted, a filter's organism retentivity was signified by the symbol T_R meaning "Titer Reduction." It referred to the ratio of particles in the influent stream to those in the effluent. This is also the definition of filter efficiency (Pall and Kirnbauer, 1978). The concurrent term Beta Ratio described the filter efficiency of the removal of AC Fine Test Dust from hydraulic fluids. Reti (1977) and Leahy and Sullivan (1978) applied that term to organism retentions as well. LRV, a filter's LRV, is an expression adapted by HIMA, the Health Industry Medical Association (now known as Advamed) in an effort to standardize a code word for the predilection of a filter to retain organisms. The LRV is the logarithm to the base 10 of the ratio of the organisms in the influent stream to those that emerge in the filtrate. Thus, if a filter has an LRV of 7, it is capable of diminishing the number of test organisms by seven orders of magnitude. Expressed as a percent reduction in the number of microorganisms, the number would be 99.99999.

To determine the specific LRV of a filter, a test challenge level has to be used that will cause some passage of organisms through the filter, in order to supply a denominator for the LRV expression. Sterilizing membrane filters do not ordinarily permit the passage of test organisms. Therefore, the LRV of such filters is described solely in terms of the numerator, the influent or challenge level, as being greater than the $\log_{10}$ of that total challenge.

The "Pores"

The filtrative removal of organisms by a filtration depends primarily on the size of the organisms relative to the size of the restraining pores. Some characterization of the filter pore sizes is, therefore, indicated.

As the name "microporous membranes" implies, pores are intersperse within the polymeric matrices of these filters. The separating of particles, both organisms and others, from fluid suspensions takes place at the pore sites, the size of which determines the particle sizes retained by the mechanism of size exclusion or sieve retention.

Too little is known about the numbers, sizes and/or shapes of the pores of microporous membrane. The membrane structure is usually pictured as being analogous to that of a polymeric sponge. An hypothesized oversimplification of the pore passageways is that of irregular and tortuous capillaries that are, therefore, more extended in length than the filter's surface-to-surface thickness. The pores are marked by irregularly restricted diameters, whether at their entrances or within their channels, that provide the choke-points that interfere with particle passage. Nevertheless, however complex, the pores are conveniently pictured as being essentially cylindrical and composed of interconnected spaces extending as pathways through the depth of the polymer matrix.

A more explicit pore architecture is hypothesized wherein the pores are not columnar passageways but are rather aggregations of polyhedral open-walled chambers or cells of different proportions, the "pore sizes" of which are defined by the dimensions of polygonal apertures leading from one hollow cell to another (Williams and Meltzer, 1982). This reasoning envisages pore formation as the coming together of hollow spheres. These eventuate from the membrane-casting process whose spatial clustering is under the influence of area-minimizing forces. The phenomenon of clustering through polyhedral spatial arrangements is known from the studies of contiguous soap bubbles whose structural formation is under the same constraints (Fig. 1), (Almgren and Taylor, 1976).

According to Johnston (2003, Chap. 3), the very concept of a definable "pore" is an artificiality when applied to microporous membranes other than the straight-through columnar pores that characterize the track-etched variety. The complex geometry of the sponge-like membrane results in the pores having ratios of cross-sectional areas to perimeters, called the hydraulic parameters. These vary over the entire thickness of the membrane. A membrane's depth can be considered to be constructed of a number of

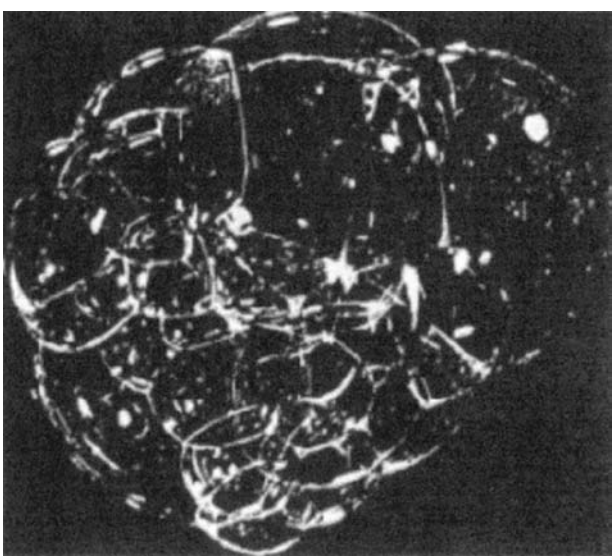


FIGURE 1 Free-floating soap bubbles.

superimposed unit planes which in their aggregate impose their effect on retention and flow rates (Piekaar and Clarenburg, 1967). These vary over the entire thickness of the membrane. (Johnston, 2003). The "pores" so considered are presumably connected throughout the unit planes to constitute pathways for fluid flows. However, where blockages by particle retentions interfere, flow redistributions may result through new "pore" alignments.

The "pores" so considered are, however, hypothetical constructs useful in understanding filter performance. Unlike the track-etched pores they are *not* integral, structural pathways for fluid flow. Nevertheless, the events taking place at the pores are depicted as if they were continuous and integral paths through the depth of the filter.

Particles are separated from the fluid by adsorptions to pore surfaces, as well as by the size exclusion mechanism. The adsorptive sequestration mechanism can serve to remove suspended matter that is smaller in size than the pores. Thus, the meaning of the average pore size as reflecting organisms retained exclusively by the size exclusion mechanism is necessarily an oversimplification.

Sieve Retention Mechanism

As noted, in sterilizing filtrations, for the drug preparation to emerge as a sterile effluent, the filter needs to have the capability of withstanding the direct confrontation of 1×10^7 CFU of *B. diminuta ATCC-19146* per cm^2 of EFA in a standard test. This accords with the FDA's concept of a "sterilizing filter" (FDA, 1987, 2004). The *B. diminuta* serves as a model organism for the microbes likely to be encountered in pharmaceutical contexts.

Filter selection is made essentially on the premise that sieve removal is the exclusive mechanism of particle retention; adsorptive effects, being difficult to predict, are ignored. It is conveniently assumed that even the filter's largest size pores will restrain the passage of the smallest of the challenging *B. diminuta* organisms. The correlation of a filter's bubble point value, related to the largest size pores, with its quantified capability to retain the *B. diminuta* test organisms is experimentally determined by the filter makers using a standardized test.

The filter's classification as suitable for a filtrative sterilization is postulated on this relationship. The filter's pre-use bubble point and/or diffusive flow value is assessed by the user although its level is already known from measurements made by the filter fabricators. Filter manufacturers integrity test each sterilizing grade filter cartridge prior to release. Nevertheless, the user may be obligated by the regulations to confirm the identity and propriety of the particular filter selected for use. The integrity of the filter is affirmed by the post-use test's value.

THEORETICAL BASIS FOR THE INTEGRITY TESTS

The integrity testing of a hydrophilic filter depends upon the interpretation of the flow rates of a test gas issuing from the downstream side of its water-filled pores exposed to its differential pressure at successively higher applied upstream pressure levels. The endpoint sought is the differentiation of a diffusive airflow from that of a viscous or bulk airflow. The measurement relied upon to recognize the distinction is the bubble point, so called because its appearance is manifested by a stream of bubbles. Its value in the pressure terms of psi or bars is individual for each of the various filter types, and for the pore size rating classifications within each type. The former derive from the filter type's particular molecular composition; the latter, from the pore's restricted diameters. This

appertains also to the integrity testing of hydrophobic filters using liquids of lower surface tensions such as aqueous alcoholic solutions in place of water as the wetting fluid.

Consider the course of a procedure involving a progressive increase in the differential pressure of a gas upon a water-filled membrane. The resulting increasing rate of gas passage through the filter will progress in stages. The initial gas flow will occur by diffusion through the water-filled pores. This will be followed by a bulk or viscous airflow through pores blown empty of water at the bubble point pressure. This point marks the transition from diffusive to viscous airflow. It identifies the largest pores of the filter by way of the lowest differential pressure needed to blow them clear. The implications are to the smallest organisms the filter can retain by sieve retention. It is the largest pores that are emptied first. The next smaller size pores follow progressively. In agreement with the bubble point equation, they exhibit the inverse relationship of the bubble point pressures to the sizes of the largest pores.

The various filter types differ in their bubble points because their particular molecular structures require dissimilar and distinct force levels to separate (and expel) the water molecules from the individuality of the polymeric pore walls. The sizes of the largest pores, so revealed, are not measured in dimensional units, but are rather approximated by their inverse connection to pore size as identified by their bubble points. Correlating the various differential pressures with their corresponding organism retention capabilities (LRVs) establishes the desired integrity test. It relates a filter's putative pore size to its organism removal ability. If a filter identified by its pre-use bubble point value suffices to perform a particular organism removal, and its bubble point value measured post-use is found to be unaltered and within the manufacturer's limits, its identity is confirmed as being unblemished.

The Capillary Rise Phenomenon

An understanding of the phenomenon governing the rise of water in glass capillaries and the subsequent emptying thereof explains the relationship of a filter's largest pores to the bubble point integrity test.

Water will rise in a glass capillary tube to an extent governed by its diameter. If one of the ends of a number of glass capillaries of different diameters were dipped into water, the liquid would rise highest in the narrowest tube. The narrower the diameter of the capillary, the greater the rise. The hydrophilicity of the (polar) silicate material constituting the tube, and the polarity of the water influence the extent of rise. Water will not rise in a polyethylene (hydrophobic) tube or capillary, nor will mercury rise in glass capillaries (see section on wetting of pore surfaces).

The reason for the rise of water in glass is the hydrogen bonding that is cohesive among the water molecules. Similar partial-charge influences create an adhesive bond between the water molecules and the silicate anions of the glass. It causes the one to spread over the other. It results from the attractive forces existing between partial-charges of opposite signs. One such is situated on an atom that is part of a molecule of the glass surface; its opposite partial-charge is sited on an atom, whether hydrogen or oxygen, of a water molecule. The presence of such an intermolecular bonding action is signaled by the concavity of the liquid meniscus within the capillary. The interaction of the water and the silicate surfaces pulls the liquid film upward along the glass walls. The liquid molecules not in intimate touch with the glass constitute, in effect, a free-standing column of water. Hydrogen bonding only with one another, they are less affected by the rise in proportion to their distance from the capillary walls. The mass of hydrogen bonded water is lifted by

these cohesive forces operating within the water bulk, as expressed by the surface tension, gamma, λ, the term in the equation responsible for the capillary rise.

Within the capillary this chain of mutually hydrogen bonded molecules is manifested by a concave meniscus. The water rises until it is balanced by the opposing force of gravity. Such attractive forces are absent between water and polyethylene capillaries, or between mercury and glass because of the dissimilarities in the surface tensions of the liquids and solids. In cases involving non-wetting liquid within a capillary the meniscus is convex. This is the upper crescent-shaped part of Langmuir's spherical free-falling drop. The liquid drop falling through the air is spherical because its molecules are dissimilar in their solubility parameters from those of the air that constitute their ambient surroundings. The free surface energy of the molecules comprising the drop's surface is expressed as surface tension. This results in a sphere, the geometric form which encloses the largest volume possible within its minimum extent of surface.

Voiding the Water-Filled Capillaries

For the purpose of integrity testing it is assumed that the pores of microporous filters act as do capillaries when they are wetted with water. To expel the water from the membrane pores requires a countervailing force to destabilize the capillary rise equilibrium. At the peak of the water's height in the capillary, the upward force resulting from surface tension effects, is balanced by the force of gravity. Air pressure applied to the surface of a wetted membrane contained in a suitable holder will upset this equilibrium. Enough pressure will have to be exerted to overcome the bonding forces adhering the water molecules to the pore surfaces. At this differential pressure, the bubble point, the water will be ejected from the membrane's largest pores.

BUBBLE POINT TEST

Pore Size and Pore Shape

It is the bubble point that is relied upon to gauge pore sizes. This teaching stems from an examination of the capillary rise phenomenon. However, dimensional pore sizes cannot with accuracy be calculated from the bubble point equation:

$$P = 4\gamma \cos \Theta / d,$$

where P is the pressure required to expel the liquid from the filter pore whose diameter is d; while gamma (γ) is surface tension (test liquids other than water may be used), and theta (Θ) is the angle of wetting.

For example, assuming a pore size of 0.2/0.22 μm, the calculated theoretical pressure needed to expel a liquid like water would be 14.4 bar; at γ for water of 0.072 N/m at 20°C; and cos $\Theta = 1$. Certainly, this is not the case. The bubble point pressure commonly found for 0.2-/0.22-μm rated filter membranes is around 50 psi (3.5 bar).

The reason for the differences between the calculated and measured dimensions (or pressure levels necessary to cause liquid to be expelled from the pores) derives, as stated, from the assumption inherent in the equation that the pores are circular in cross section (see "Pore" section above). A scanning electron micrograph of a typical inverse phase microporous membrane is shown in Figure 2. The convoluted polygonal shapes of the pores make it impossible to directly measure their perimeters or the areas they enclose.

In the one instance where calculations based on the shape factor can be performed—for woven metallic cloth of the Dutch twill design, wherein the pores are

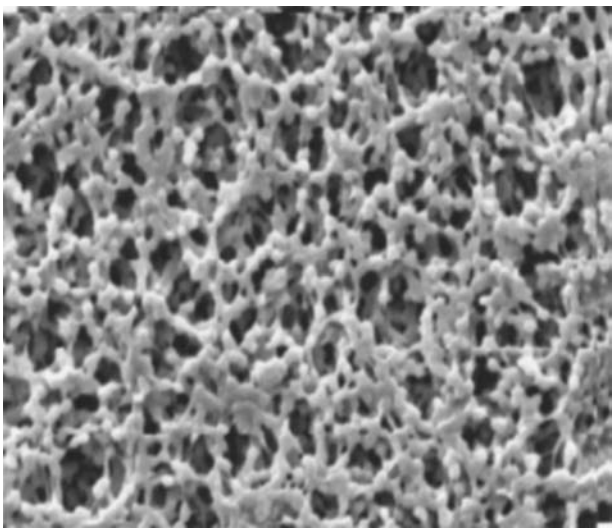


FIGURE 2 Example of quenched membrane.

skewed equilateral triangles—calculated and measured values agree (SAE, 1968). A capillary rise equation that more generally treats the shape of the pore includes a shape factor, L, for the length of the pore perimeter, and A for the area it encloses instead of $2\pi r$ and πr^2 terms correctly applicable to circles alone:

$$P = L\gamma \cos \Theta / A$$

The pore shapes importantly influence the pore diameter values. Pores having the irregular outlines that characterize membrane voids are smaller in their extent of open volume than are those of a similarly sized spherical periphery. This is so because the area within a circle is the largest expanse that can be enclosed by a given perimeter. If the same size perimeter assumes any shape other than circular, the area it encloses must be smaller. It is, thus, safe to say that particles much smaller than the numerical pore ratings will be sieve-retained by membrane filters (Ferry, 1935; Petras, 1967).

In any case, the bubble point method based on capillary rise yields only relative values of the membranes largest pore sizes. This accords with the previously quoted statement of the Aerospace Recommended Practice (SAE, 1968), "No bubble point test measures actual pore size but only allows correlation of the measured capillary pressure with some dimensional characteristics of the pore structures." Nevertheless, however roughly, the bubble point is indicative of the sizes of the filter's largest pores.

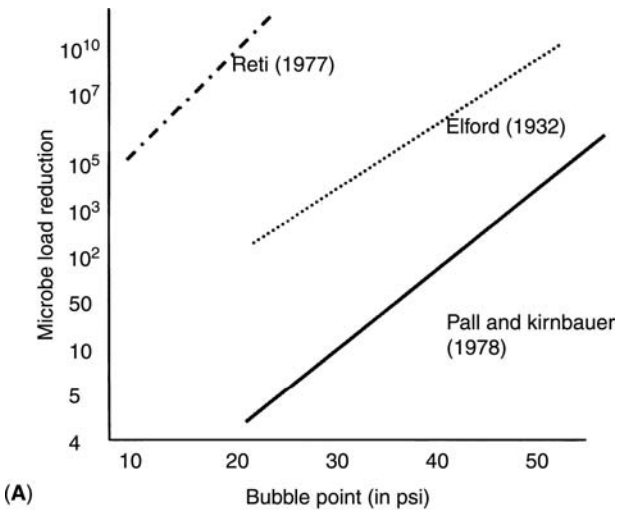
Sensitivity of Bubble Point Measurements

The bubble point is the measurement of prime interest where filters are engaged to produce sterile effluent in processing operations. For example, for filtrative sterilizations, an LRV is required of a membrane sufficient to sustain a *B. diminuta* bacterial challenge of 1×10^7CFU/cm^{2}of EFA. Pore size and organism retention are directly related. The higher the bubble point's differential pressure, the smaller is the pore (diameter), and the larger is its LRV. This relationship is illustrated in Figure 3A (Johnston and Meltzer, 1979) as a composite of plots of data correlating bubble point values and their corresponding LRVs, as reported by Reti et al. 1979), by Elford (1933), and by Pall and Kirnbauer (1978).

Data from Leahy and Sullivan (1978) are shown in Figure 3B. The correlation is evident from the straight lines having essentially the same slopes, as obtained from the four groups of data.

Figure 4 shows that a ~10% change in bubble point (and subsequent changes in permeability and pore size) results in a tenfold change in the bacterial reduction ratio. The figure posits the conclusion that when one attempts both to measure such large reduction ratios and to reproduce the results within ±10%, one should expect to see tenfold variations in the microbe reduction ratio. In other words, if 10^7 is a true value, one may find numbers anywhere between 10^6 and 10^8. An uncertainty of 10% inheres to the bubble point measurement, as it is manually conducted.

Hofmann (1984) on the basis of hypothesized dimensions calculates that the bubble point's detection for a 47-mm-disc will involve a gas flow rate of ~20–50 µl/min, equivalent to a linear gas flow of 6–16 mm/min within the 2-mm diameter tubing



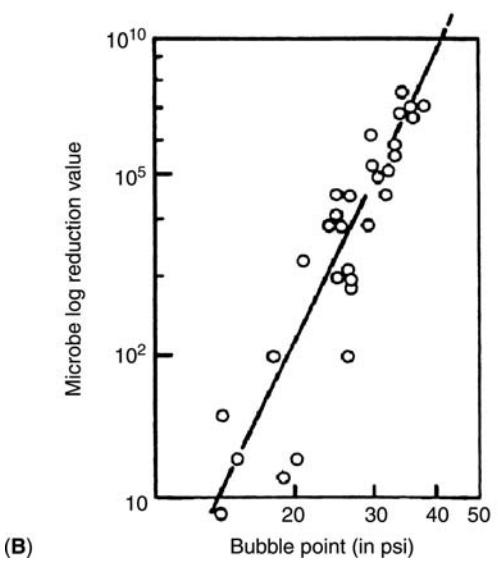


FIGURE 3 (**A**) General curve relating organism retention to bubble point. *Source*: From Johnston and Meltzer, 1979. (**B**) Correlation of bubble points and organism retention. *Source*: From Leahy and Sullivan, 1979.

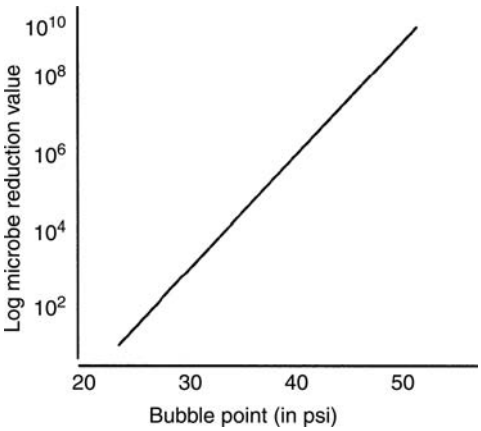


FIGURE 4 Correlation of bubble points and organism retention from data reported in the literature. *Source*: From Johnston and Meltzer, 1979.

downstream. To attain the same measurement sensitivity for a 296-mm diameter disc, the same viscous gas flow of 20–50 µl/min would be involved, in addition to the diffusive background flow of 1.5 ml/min. To achieve the same sensitivity for a 10-inch sterilizing grade cartridge, the detection of the 20–50 µl/min bulk airflow against a diffusive gas flow background of 12 ml/min would be required; clearly not possible with the present instrumentation.

Measuring test points on a curve generally decreases the accuracy of their readings. This is one reason for not measuring the differential pressure points at above 80% or so of the bubble point. Accordingly, the bubble point's identification of the filter's largest pores is tenuous, as also is the uncertainty of its location on the curve. Given the importance of the bubble point's indication of the differential pressure level that motivates bulk airflow, its position on the curve and the differential pressure that quantifies it requires a more sensitive evaluation than is available. Hofmann (1984).

The pursuit of a more exact locating of the bubble point by way of identifying its differential pressure level led to an investigation of other testing technique, and to subsequent efforts at improving test operations. The diffusive flow integrity test, soon to be discussed, was designed to integrity test filters based on a single-point analysis.

Bubble Points: Perceived and Intrinsic

The gas passing through the filter enters a pool of water and is made visible as a stream of bubbles (Fig. 5). Hence, the name "bubble point." In manual testing the *intrinsic* bubble point is likely not to be noticed immediately. The mini-quantities of air emerging from solution must first collect in sufficient quantity to form the bubbles. What is first noticed is the *perceived* bubble point; the exact instance at which the bulk air becomes apparent to the eye or automated test instrument. It is probably always at a higher differential pressure than the intrinsic point whose early detection requires great sensitivity. The larger the filter area, the less accurate and the more subjective the bubble point becomes. The so-called bubble point, perceived at a higher pressure, is taken to represent a pore smaller than it really is. Organism retentions based on particle size/pore size relations, therefore, gain an undeserved but helpful safety margin derived from the mistaking of the perceived bubble point as being the intrinsic Johnston et al. (1981) found that a 7-member panel differed markedly in identifying the perceived bubble points of 10 inch cartridges.

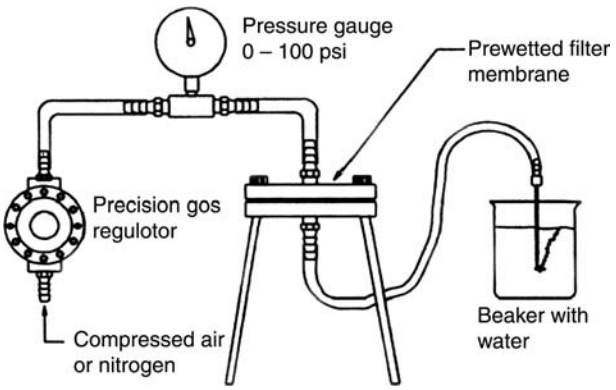


FIGURE 5 Manual bubble point test system. *Source*: Courtesy of PDA, Bethesda, MD, 1998.

The range of values varied between 5 and 50 mL/min of gas flow. A similar range, 20–70 mL/min was found by Hofmann. This equaled an increase of 10–60 mL/min over the 10 mL/min diffusive flow expected at that differential pressure level. At so insensitive a detection level, a 10% departure from the intrinsic value would result in an error of two orders of magnitude in the LRV. If the desired LRV of a filter is 7, the bubble point reading would be between an LRV reading of from 6 to 8. This bespeaks the strong dependence of obtaining useful results from the fine tuning of measurements. The automated test machines do incorporate instruments of greater sensitivity.

The Knee Area of the Curve

The curvilinear trace, the knee area of the curve just discussed, is important enough to merit a sharper focus. It represents a mixture of air originating from both diffusive and viscous or bulk air passage. The initial break in the straight line's diffusive airflow leading into the knee curve occurs when the applied differential pressure is strong enough to force water from the group of largest filter pores in accord with the teachings derived from the voiding of water-filled capillaries. This is the bubble point. It marks the beginning of bulk air passage. As the differential pressure increases successively, it is followed in series by the voiding of the next-smaller set of pores, etc. until all the pores are blown empty of the liquid. This causes successively increasing rates of air exiting the filter as a bulk flow, in addition to the diffusive flow that remains ongoing until all the pores are emptied of water. The curvilinear trace after the bubble point, the knee of the curve, is important enough to merit a sharper focus. It consists of a mixture of air originating from both diffusion and bulk or viscous passage.

The breadth of the curvilinear region is largely the product of the filter's pore size distribution which presents a spectrum of pore sizes, the emptying of which occurs stepwise over a span of increasing differential pressures. The wider the distribution, the more extensive the area of curvature. Membranes of narrower pore size distributions show sharper transitions; both from the diffusive airflow to the emptying of the pores, and from that to the completely viscous airflow at still higher differential pressures (Pall and Kirnbauer, 1978). However, the presence of anisotropic pore structures, more easily voided, also contribute by diffusion to the increasing airflow, and confuse the recognition of the "true" bubble point, and its exact initiation point.

What is desired is the unambiguous identification of the bubble point, described in differential pressure units. This requires knowing exactly when bulk air passage

commences. However as detailed above in Section: Diffusive Airflow Influence on Bubble Point, at that very point the diffusive airflows from filters having an EFA larger than about that of a 47-mm-disc obscure the sharp edge of the bulk airflow's beginning. Adding to the uncertainty is the diffusive flow contributed by the anisotropic pores. It is more likely that the thinning of the water layers in the anisotropic pores occurs earlier at lower differential pressures than the emptying of the largest pores. This occurrence would speed the rate of diffusion and add to the bubble point's obfuscation. Therefore, diffusive flow testing is preferred over bubble point when integrity testing asymmetric membranes.

Nevertheless, because of practical considerations it is common to assume that the initial break in the straight diffusive airflow line marks the bubble point. It is so assumed by the automated testers where the sensitivity of measurement is superior to that of the manual assays. The assumption focuses and makes more evident the curve's break from the linearity of its upward progression. At this point, the filter's bubble point value has increased to where it signifies smaller pores, and its correlated LRV level has also increased to where the potential for organism passage is no longer possible for reasons of size interferences (Fig. 6).

Concerns that the bubble point's arbitrary placement at the onset of the curve may lead to improper judgments and/or actions, are assuaged by the safety margins added to the experimentally defined bubble point level by the filter manufacturers. The membranes thus characterized are of a somewhat tighter diameter. They are, therefore, more retentive of smaller organisms. They do exhibit some reduction in flow rates, and are likely more restrained in their throughputs against more heavily loaded suspensions. Users are obliged to respect the filter manufacturer's stipulated bubble point values in their membrane applications.

Normalized Bubble Points

Interestingly, the bubble point of a given type filter may upon measurement seem to increase as the filter's effective or available filter area decreases. Johnston and Meltzer (1980) illustrated that this is an artifact occasioned by the larger volume of air produced per area of filter by a given differential pressure. This quantity of air, being larger, is earlier perceived as bubbles, and is, therefore, earlier identified as the bubble point. Figure 7 shows that although the perceived bubble point does seem to differ reciprocally

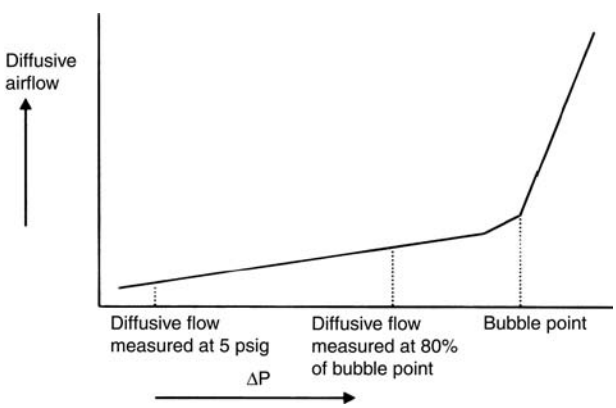


FIGURE 6 Diffusive air flow at different pressure settings.

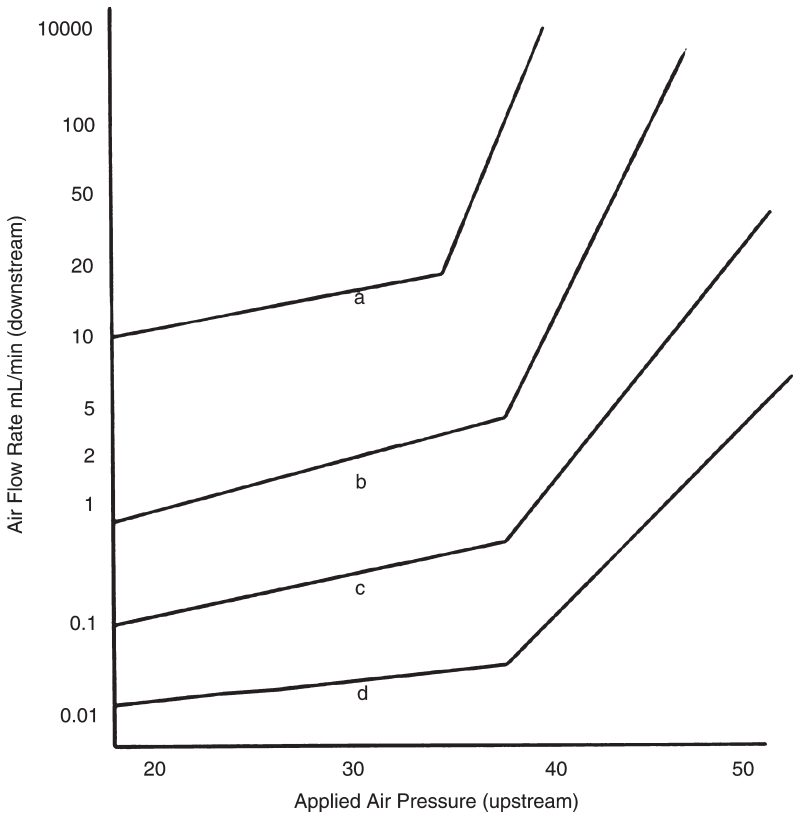


FIGURE 7 Shift in bubble points as a function of filter area for different areas of a 130 μm thick, 0.2 μm rated membrane: a) 4545 cm^2 in a pleated cartridge filter; b) 589 cm^2 in a 293 mm diameter disc; c) 44 cm^2 in a 99 mm disc; d) 9.6 cm^2 in a 47 mm disc. *Source*: From Johnston et al., 1981.

with the filter area, the inherent bubble point of the membrane type wetted by the same liquid remains constant. Dividing the apparent (perceived) bubble point values by the filter areas serves to normalize the data, converting it to its inherent value. However, in practice bubble point data are seldom normalized. Instead, the bubble point is simply reported along with the size of the filter for which it was obtained.

The Bubble Point's Significance

The basis for the integrity testing of filters depends upon the interpretation of airflows through membranes wetted by water. The bubble point measurement identifies the largest (diameter) pores present in a membrane by the differential pressure required to empty them of their water. The largest (diameter) pores are vacated first. The pores thus sized are correlated with their ability to restrain the passage of organisms. It is this correlation that enables a filter to be characterized and selected for an application requiring a given pore retention value. The differential pressure supplies the work function necessary to disrupt the bonding between the water molecules and the molecules of the hydrophilic pore surface that constitutes wetting, and to forcibly separate the water from the pore walls. The bonding strength of the wetting interaction to be overcome is peculiar to each

particular filter/fluid combination, and is different for each pore size rating of each of the different type filters.

Given that the dominant mechanism of particle removal by filters depends on size discrimination, the concern in conducting a sterilizing filtration is that the filters should not have pores large enough to enable organisms to escape capture. To address this concern a filter's pore sizes should be known. However, the filter manufacturers' pore size designations may not do because two filters labeled identically, each by a different filter manufacturer may, in fact, not be so. In the absence of industry standards, pore size ratings are assigned by the filter purveyors on an individual basis. Filters from different manufacturers may bear identical pore size labels, but, regardless, may differ and cannot really be compared on the basis of their assigned ratings. The bubble point technique offers a different approach to filter sizing in that it quantifies a filter's largest pores in specific differential pressure units.

Consider: By definition, groups, however assembled, differ from one another more greatly than do members within a group. Thus, the filter groups classified by pore size ratings do differ from one another. But the individual members of each group are also unalike, albeit to some lesser amount. Hence, such descriptions as: An "open" 0.2/0.22 μm, or a "tight" 0.2/0.22 μm. Both bear the same pore size label, but do differ to an extent that may be significant in a given application. Therefore, comparing two filters of a given filter type and pore size classification involves some uncertainty. But comparing two filters in terms of their "largest pores," each identified individually by a specific differential pressure value, is a less ambiguous situation. This is an advantage of the bubble point designation. Its comparisons among filters of a given type are more credible (Jornitz et al., 2007).

However, the comparison of organism retentions among different types of filters based on bubble point values remains elusive. The number of their pores, their sizes and size distributions, and especially their pore shapes may not be assumed to be the same.

The Largest Pores: What They Portend

The utility of the bubble point derives from its identifying a filter's largest pores as measured by the differential pressure required to expel the water from a wetted filter. The flow of bulk air through the emptied pores produces the stream of bubbles that marks the endpoint. The bubble point equation follows:

$$P = 4\gamma \cos\Theta/\mathrm{d}$$

P is the pressure required to expel the liquid (test liquids other than water may be used) from the filter pore whose diameter is d, while γ is surface tension, and Θ is the angle of wetting. The integer serves as a correction factor necessary for the better conformance of the equation with actual measurements. Its presence acknowledges our imperfect understanding of the phenomena involved.

Laplace's Law is descriptive of pores with the shapes of ideal cylinders. Nevertheless, as applied here, it makes plain in equation form the inverse relationship of P, the bubble point pressure, and d, the pore's diameter. The bubble point value is the applied differential pressure level just sufficient to expel the water from the largest pores. However, the value calculated by way of the Laplace equation is always lower than would be expected. The differences are ascribed to imperfect wetting of the pore surfaces. This increases theta, Θ, the angle of wetting, and affects the results. The differences also

may be due to the irregular shapes of the pores, and the pore size distribution. As said, the bubble point equation applies to regular cylinders, not to the irregularly shaped pores of the microporous membranes.

Different type filters may differ in their completeness of wetting, in their pore size distributions, in the shapes of their pores, and even in their pore sizes as classified by the non-standard systems of the individual filter manufacturers (Hofmann, 1984). Consequently, the bubble point is not an absolute measure of a filter's largest pores. Comparisons of bubble point values among filters of different type but of identical pore size ratings do not lead to useful results. However, the bubble point does serve, for the same type of filter, as a relative measure of its largest pore size against which retention data can be correlated (Johnston et al., 1981).

Hofmann (1984), making enabling simplified assumptions, details mathematically the sensitivity with which differential pressures and flow volumes must be measured to differentiate between the diffusive and bulk airflows contributing to the total quantity of air passing through a wetted membrane. The precise identification of the bubble point depends upon the sensitivity in measuring differential pressures, and/or gas volumes. This is best achieved using automated test machines.

In the striving for exactness during the development of the bubble point test, different approaches were advocated and explored for their possible advantages. The presently accepted integrity tests arose from such efforts.

Diffusive Airflow Influence on Bubble Point

The bubble point measurement can be replicated at multiple differential pressure points in ascending order. The plot of this series of test points, each of which represents the rate of *diffusive airflow* as a direct function of its particular differential pressure, produces an upward-trending straight line of moderate slope. It extends to some level above the differential pressure value attained at about 80% of the bubble point level. Above this point the line begins to curve upward. This is taken to mark the bubble point. At its advent, bulk airflow commences through the largest pores emptied of water by the attained differential pressure. The airflow is then a mixture of bulk air plus the air diffusing through the smaller pores still filled with water. What is sought is the exact point when bulk airflow begins. However, the accuracy of the measurements is clouded by the simultaneously occurring large diffusive airflows. For small area filters (EFA), for example, flat 47mm discs, the visual sensitivity of manual determinations suffices to define the bubble point. The diffusive airflows of larger area filters overwhelm the bulk airflow, and obfuscate the bubble point (Johnston et al., 1981).

According to Hofmann (1984), the transition pressure, where the increase in the rate of gas bubble appearance ceases to be proportional to the incremental increase in challenge pressure, necessitates "subjective" bubble point determinations.

The bubble point differs for each pore type. This reflects the different and specific molecular composition comprising the filter, and the strength of its adhesive bonding to water molecules. Thus, various combinations of liquids and solid surfaces coupled in the wetting action result in distinct and different bonding strengths. The various pore size ratings of a given filter type also differ in bubble point readings because the narrower the pore, the greater the force necessary to expel its liquid content. The differential pressures are needed to surpass the wetting interaction, the cohesive bonding strength, between the solid molecules of the pore walls and those of the liquid. Attaining the differential pressure level of a wetted filter's bubble point results in its largest pore's being emptied of the water. The result is a clear channel available to bulk airflows.

Advantages and Disadvantages of Bubble Point Testing

As for every test methodology, there are benefits but also limitations to the bubble point test. This certainly is also valid for the other described integrity test methods.

Advantages

The bubble point test can be directly correlated to membrane pore size. It detects the largest pore by forcing the liquid from such pores and creating a bulk air flow which can be detected. It is relatively easy to perform on small to medium scale filters. It is the only test, which can be performed on small scale filtration devices, which cannot be tested via the diffusive flow measurement or the pressure hold test. The test duration can be brief due to short stabilization periods and faster pressure rises. The correlation between the bubble point and the bacteria challenge, as well as the water wetted bubble point and the product-wetted bubble point is reliably and easy to establish. Temperature influences are not as critical as for the diffusive flow or pressure hold test. The temperature influence restricts itself to the surface tension of the wetting medium. Usually a temperature increase of the air upstream volume does not have such an effect as in diffusive flow or pressure hold testing.

Disadvantages

When performing a manual bubble point test, one has to manipulated the downstream, i.e., filtrate side of the filter, which one wants to avoid. Furthermore, a high degree of the test person's subjectivity is involved, when the test is performed manually. Both such disadvantages can be avoided, using automated integrity test machines. The sensitivity of the bubble point test decreases with increasing filtration area, due to the fact that the diffusive flow may complicate the bubble point's detection. The use of the bubble point becomes more critical with smaller pore size rated filter membranes. The bubble point values of such pore size ratings may exceed the maximum differential pressure that such membranes can sustain, or may be above the allowable operating pressure of the filter. Bubble point measurement does not take the membrane thickness into account. This can be critical for the retentive capabilities of such membranes (Pall and Kirnbauer, 1978).

DIFFUSIVE FLOW TEST

Fick's Law of Diffusion

The diffusive airflow is an expression of Fick's law of diffusion (Reti, 1977; Waibel et al., 1996; Jornitz et al., 1998):

$$N = D\,H(P_1 - P_2)\rho/L$$

where N is the permeation rate, D is diffusivity of the gas in the liquid, H is solubility coefficient of gas in the liquid, L is the membrane thickness; $(P_1 - P_2)$ is the differential pressure; is total porosity.

The diffusive flow permeates the pores of all sizes, large and small. It should be noted that the sizes of the pores do not enter into the equation; in their aggregate they comprise L, the thickness of the liquid layer, the membrane being some 80% porous. The critical measurement of a flaw is the thickness of the liquid layer. Therefore, a flaw or an oversized pore would reflect the thinning of the liquid layer due to the elevated test

pressure on the upstream side. The pore or defect may not be large enough for the bubble point to become manifest, but the elevated test pressure will produce a larger diffusive airflow.

Besides the thickness of the liquid layer, the effective filter area is a critical parameter for diffusive flow measurement. The measurement of the diffusive flow will be impossible at a too small filter size, because the restricted filter area would not allow enough air volume to be diffused through the wetting liquid layer to be measured accurately. Therefore, a filter element that shows less diffusion will not necessarily retain better. It may mean only that this filter element contains less membrane area or that the membrane has a reduced total porosity, or that it is thicker than stated.

Forward Flow Test

There is a twofold phenomenon attendant upon a differential gas pressure affecting the liquid filled membrane. As discussed previously (see The Knee Area of the Curve), under pressure the fluid is eventually expelled from the largest pores as the bubble point is reached. Initially, the gas supplying the pressure dissolves in the liquid in accord with Henry's law that states that a gas dissolves in a liquid to an extent defined by its partial pressure over the liquid. The dissolved gas diffuses to the low pressure side of the filter from where it leaves the solution as a diffusive airflow.

The rate at which the gas leaves the filter, whether by diffusive or bulk flow, depends upon the applied differential pressure. As discussed, the line traced by plotting the rate of the exiting gas against the delta P describes a straight course until a point is reached at which it begins its curvilinear upturn to a straight line segment. At that point the flow is only of bulk air; all the pores having been emptied of water. The bubble point lies somewhere on the curve. It represents the bulk airflow which at that point enters and mixes with the diffusing air. The initial linear portion of the trace describes the diffusive airflow that leads to the bubble point. Like the bubble point, it can serve as an integrity test.

If the test point for a newly assayed filter melds into the diffusive airflow line previously established by multipoint testing as being characteristic of that filter type, then the filter's integrity is confirmed. A higher diffusive airflow than expected is interpreted as a failed integrity test due to a flaw or to a larger pore that is untypical for that type membrane. A lower rate of diffusive airflow signifies structural aberrations in the filter, whether thicker membrane, a lower porosity, some unforeseen pore blockage, etc. Thus, the single-point serves as a positive integrity test for that filter type.

The original test point selected by Pall at ~5.25 psi (0.35 bar) was changed to a differential test pressure of about 80% of the bubble point level (Reti, 1977). The alteration increased the test's sensitivity. Hofmann (1984) reports that testing at a differential pressure of 2.7 bar instead of at 320 mbar increased the test sensitivity twenty-fold.

Dr. Pall (1973) observed that the straight line diffusive airflow portion of the trace that led to the filter's bubble point established its values as being a function of the differential pressure levels at which they were measured. This seemingly invariant relationship, it was assumed, characterized but was different for the various given types of filters. Once quantified as having a particular constant slope and reach, a given straight line, can be identified equally well by single point as by multipoint plotting. Dr. Pall suggested that measuring the airflow values at any differential pressure point on the linear line along with its bubble point could serve as an integrity test. Dr. Pall named it the

forward flow bubble point test. It is usually referred to as the forward flow test. One of its claimed advantages is that it eliminates the burden in time and effort expended in the multipoint testing used to demonstrate that the line leads directly to the bubble point.

Two measurements are called for. The first should show that the filter being assayed yields a point that coincides with the straight line typical of integral filters of that type, as established by prior multipoint testing. The second is the bubble point itself. The integrity of the filter is confirmed by these two measurements.

Some experimenters in conducting the test believe that it is unnecessary to measure the bubble point itself, assuming that the slope of the line inevitably leads to it. However, the authority of the single point depends on its demonstrated positioning on the diffusive airflow line established by prior multipoint testing. Measuring the single point at a given delta pressure level reveals nothing about the filter's behavior beyond the test point. Assumptions regarding the outcome may be expedient, but require the sanction of validation to be acceptable.

Single-Point Plus Bubble Point

An obvious advantage of the single-point test is the time and effort saved in making diagnoses, a worthwhile goal, especially in process filtrations. However, added advantages may be available to those who still prefer the routine of multipoint analyses, but who desire the speed of single-point testing. Schroeder (2001) questions whether multipoint airflow integrity testing need entail excessive time investments. He suggests that the straight line reliability of multipoint testing can be obtained by utilizing two test points instead of the one single-point. The point of origin at zero differential pressure already exists. To this initial point would be added the single point being discussed, plus measurement at the bubble point.

The flow rate is a function of a filter's total porosity. The single-point testing is measured at the differential pressure level equivalent to 80% of the bubble point. It is based on the assumption that its results would fall on the straight line that would have been traced to the bubble point level characteristic of integral filters of its type had the testing been performed at multipoint pressures. The assumption is unwarranted. Were the test point to fall below the line it would indicate a tighter membrane; above the line would signify a failed filter.

Testing at 80% of Bubble Point

In proposing the forward flow bubble point test, Pall had performed the testing at the differential pressure of 5.25 psi. Presumably, any differential pressure would suit as well for in-process testing given that it is a point on the straight line, as are all of the multipoints composing the plot. All the differential pressure points are equivalent in this regard.

As can be seen from Figure 8, however, absent bubble point testing, integrity failures may escape detection. Testing only at about 5 psi could disclose nothing about the filter's integrity at pressures above that level. Failures would not be disclosed unless the bubble points were also assessed as points of reference. It was realized that without actually assaying the bubble point, assuming a straight line from a point so far removed from the bubble point entailed far more risk than one performed as close to the bubble point as possible (Reti, 1977). As a result, the single-point selected for testing was adjusted to ~80% of the bubble point's differential pressure level; as close as possible to the break in linearity, while avoiding the uncertainties of measuring on a curve

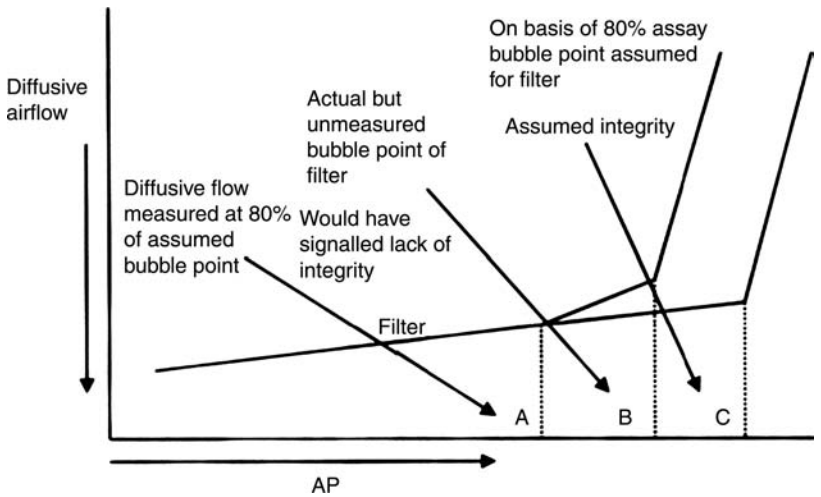


FIGURE 8 Diffusive airflow at different pressure steps.

(Reti, 1977). The advantages of single-point testing can perhaps best be applied when the multipoint line against which the single test is referenced is already available. Attained when the line extended to the bubble point has already been defined for the filter type.

Correlation of Diffusion and Retention

As in bubble point measurement, the numbers obtained from diffusive airflow tests have significance only because they can be correlated with organism retentions. The measurements are usually stipulated to be performed by the filter manufacturer, at the defined test pressure that was used in securing the correlation with organism retentions, usually at ~80% of the bubble point value.

The diffusive airflow integrity test does not confine its measurements solely to the set of largest pores or through a single flaw, as does the bubble point. Rather, it measures the airflow diffused through all the available pores. A larger pore or flaw will be evidenced by a higher total diffusive flow, possibly over the allowable limit set by the bacterial correlation itself. A higher rate of diffusion caused by the differential pressure then being applied may also be due to an anisotropic effect. At higher differential pressures, a thinning of the liquid layers in the larger inverted "V" shaped, or downstream—pointing funnel-shaped pores takes place. These are inevitably present to some extent in cast membranes (Kesting, 1985). These thinner aqueous barriers are more readily diffused.

For reasons of prudence, filter manufacturers commonly add a safety margin onto the point of bacterial breakthrough, the true correlation point. The resulting limiting value is the minimum diffusive airflow level described in the user's manual or validation documentation. It is required, however, that users observe and respect the limits of these non-destructive tests, the safety margin included.

Advantages and Disadvantages of Diffusive Flow testing

Advantages

The diffusive airflow test has a high sensitivity which increases with larger filter surface areas. A thinning of the liquid layer takes place in the larger pores or flaws as the differential pressure mounts. This results in an increase of the diffusive flow. The diffusive flow

measures the entire pore volume of the membrane matrix, including flaws, if any. The test pressures of the diffusive flow assay can be sensitive enough to identify the above mentioned thinning of the wetting liquid layer within a larger pore. This anisotropic effect may not be detectable with the Bubble Point test, but will be detected by the diffusive flow test, especially when automated machines are used. Furthermore, the diffusive flow test is very well suited for membranes with small pore size, for example, 0.1 μm rated filters. If one uses the Bubble Point test for such filter pore size ratings, the maximum operating or differential pressure the membrane can sustain may be exceeded and the filter damaged. In such cases it is advisable to use the diffusive flow test.

Diffusive airflow measurements have their advantages in addition to serving as indicators of filter integrity. They can more precisely reveal filter incompatibilities. They can be used to gauge the completeness of a filter's wettability, and they are more revealing of a filter's clean water flow properties. Usually smaller pores are wetted with more difficulties than larger pores. This would not be detected by the Bubble Point measurement, but can be evaluated by the diffusive flow test.

Additionally, the diffusive flow test can be correlated to the Bacteria Challenge test as described in the paragraph above. Such correlation is easier to accomplish, when the filter's pore size distribution is narrow. The correlation exercise is then very well defined and easy to perform.

Disadvantages

Being more sensitive at testing larger filtration areas creates a problem in itself, in testing multi-round filter housings. In an effort to provide a safety margin against too open filters, the filter manufacturers may fashion cartridges that have diffusive airflows of less than the maximum acceptable rate, say 15 mL/min at the given test pressure. Consider an assembly of nine 10-inch cartridges. Assume that eight of these elements have acceptable diffusive airflows of 10 mL/min but the ninth one, lacking integrity, has a diffusional airflow rate of 55 mL/min. The total diffusive airflow rate for the nine-element assembly would be 135 mL/min; undistinguishable from the 135 mL/min expected for the integral nine 10 inch cartridge arrangement. The safety margin allowed for, in this case, masked a flawed filter.

A single-point diffusive airflow determination would not reveal the presence of the flawed cartridge. However, the slope of a straight line drawn from multipoint diffusive testing could make evident a single filter's flaw even within a multi-element arrangement (Waibel et al., 1996; Jornitz et al., 1998). The diffusive airflow rate differs directly with the differential pressure, whereas the bulk airflow, as through flaws, reflects also the fourth power of the pore radius. Therefore, at higher pressure levels the flows through the flaw would increase markedly in deviating from the straight line of the diffusive airflow. If, consequently, the diffusive airflows are plotted from multipoint pressure data, the rate of climb of the resulting curve would reveal in its increase what a single point cannot. The increased slope of the linear section of the multipoint diffusion curve would allow for experimental investigation. Such evaluation of the slope can only be done by multi-plotting the entire graph through to the bubble point.[b]

[b] This is also the basis for the flow ratio test advocated by Hofmann (1984) for distinguishing by way of the diffusive airflow an integral filter from one that is flawed. The gas flow is determined at a high pressure, 2.7 bar, and at a low pressure, 0.5 bar. When the ratio of the high pressure diffusion is compared with that of the low, the non-integral filter is seen to have the higher ratio (see the section on Flow Ratio Test).

Due to their sensitivity, temperature deviations during the test's duration will have a high impact on the result. Any temperature influence during the test should be avoided and eliminated. Even touching a hand to the filter housing or keeping such close to an air conditioning system may affect the test result. For this reason automated integrity test systems plot diffusive flow or pressure-drop, which will reveal the occurrence of deviations during the test.

Automated integrity test machine have also the advantage that the diffusive flow test can be performed from the upstream side of the filter system. The manual diffusive flow test has the disadvantage of a downstream, filtrate side manipulation, which carry the risk of a secondary contamination. Furthermore the manual test lacks accuracy, compared to an automated test system.

MULTIPOINT DIFFUSIVE FLOW TEST

Multipoint Integrity Test

The gas flow being discussed is motivated by differential pressures, which can be arranged in ascending order. The airflow is proportional to the differential pressure. In the case of a multipoint diffusion test, the points plotted in pressure units, psi or bars, against flow rates, in ml/min, trace a straight line having a moderate upward slope until at a point it departs from its linearity to begin forming an upward curve. Until this point, the airflow consists totally of air diffused through the water-filled pores. At this point, or approximate to it, bulk airflow joins the effluent air stream. The benefit of a multipoint diffusion test is the fact that one can test multiple filters and check their diffusive flow behavior precisely by reviewing the slope of the line achieved. The multipoint diffusion test finds its use in determining product-wet diffusive flow test points, or in testing multi-round filter housings. The multipoint test makes apparent the trend developing during the progression of the diffusive airflow towards the bubble point. This presents an advantage when investigations of failures are undertaken.

Anisotropic Pore Structures

In instances, there may be uncertainty about the origin of the increasing air flow rate that initiates the break in the plotted line. It need not necessarily be the bubble point onset. It may be the result of the partial voiding of anisotropic pores (Reti, 1977). Anisotropic pore structures, according to Kesting (1985), are inevitable accompaniments of the membrane casting process. These funnel-shaped pores, if pointed downstream, thin their water contents under increasing differential pressures; thereby augmenting the rates of gaseous diffusion (Figs. 9 and 10). The multipoint diffusive airflow plots of these filters, by their deviations from the linear diffusive airflow line, reveal the presence of anisotropic membrane structures. The bubble point may not distinctly be differentiated from the anisotropic contribution. Both occurrences cause deviations from the straight diffusive airflow lines. The ejection of water from pores at the bubble point would seem logically to follow the anisotropic thinning of the water barrier within the pores. Each of these occurrences marks departures from the strictly diffusive airflows. The automated test machines signal these departures from the linear as being the bubble point. This assumption, possibly unwarranted, causes no problem as regards the correlation with organism retentions, given the modification of the bubble point values by the safety margins provided by the filter manufacturers.

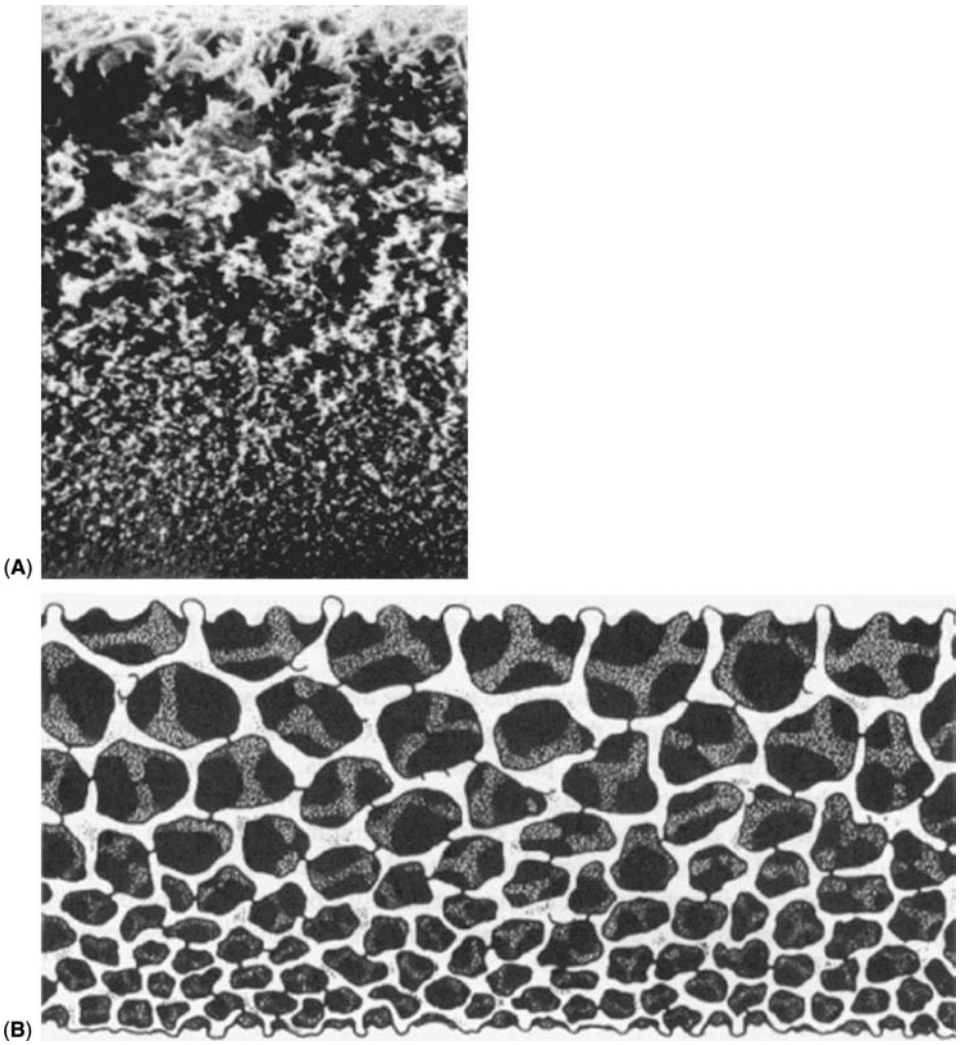


FIGURE 9 (**A**) SEM asymmetric membrane depth. (**B**) Anisomorphic membrane filter.

Distinguishing Airflows: Diffusive and Bulk

Diffusive airflows can be distinguished from those resulting from flaws. Multipoint log/log plotting will show an ascending straight line of slope 1.0 for the diffusive flows. They are proportional to the differential pressure, whereas flow through a flaw increases non-linearly with the test pressure. Bulk gas flow is proportional to the test pressure, but also to the ratio of absolute test gas pressure to atmospheric pressure. Judging a filter's integrity requires a distinction to be made between the two types of flow; the bulk flow must be sufficiently above that resulting from diffusion to be quantified. Helpfully, the difference between the diffusive and viscous airflows increases with the applied test pressure. Hofmann found that the change in test pressure from 320 mbar to 2.7 bar increased the sensitivity of the test by more than twenty-fold.

Nonetheless, according to the findings in one study, single-point testing dealing with an additional bulk flow of 1.5 mL/min due to a flaw, was not at 2.7 bar sensitive

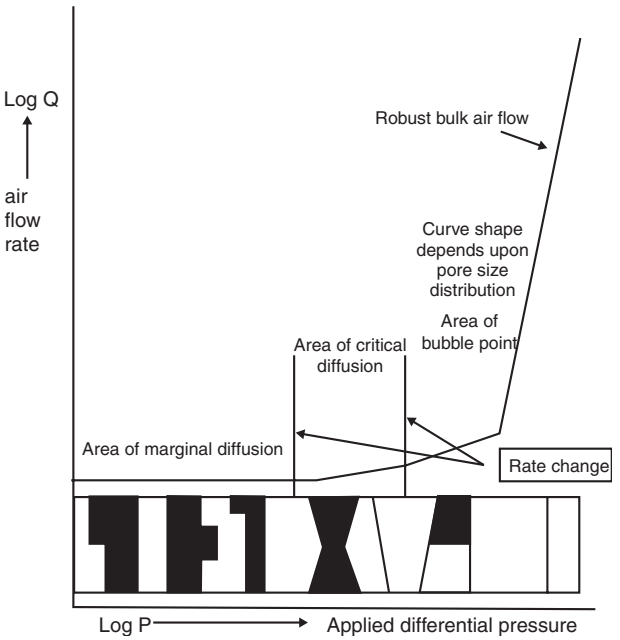


FIGURE 10 Rate of airflow through wetted membrane. *Source*: From Meltzer, 1987.

enough to discriminate between the perceived and actual bubble points (Hofmann, 1984). He suggests the application of a flow ratio test to address the problem.

Flow Ratio Test

With respect to the sensitivity of differentiating between viscous and diffusive airflows, Hofmann (1984) champions a "flow ratio test." His advocacy is based on the diffusive airflow rates being linear, whereas the bulk airflow through a flaw is not. He suggests that a most certain way of ascertaining whether a filter is flawed is to measure its diffusive airflow at a low differential pressure followed by a measurement at an elevated differential pressure. If the flow at the higher differential pressure is not proportionate to the increase in the test pressures, then a *flaw* is indicated. Pores larger than usual for the type filter should be apparent at any differential pressure from the airflow being greater than customary. However, to achieve discernable and disproportionate flows from the high and low differential pressure tests, larger test pressure differentials should be invoked. The larger the pressure difference, the higher the sensitivity of the flaw detection.

Comparing Slopes of Diffusive Airflow Lines

The airflow is the product of structural features common to all filters, namely, the porosity and characteristics of the pores; their numbers, lengths, widths, and points of constriction. These are the very structural features that characterize the several membrane types regarding particle retentions, flow rates, and, where relevant because of particle content, throughputs. Differences in these structural features create distinctions among the membrane types. The airflow lines are constructed of multipoints, each of which reflects a particular mix of these very same features. The sum result is revealed in the slope of the line. Therefore, the comparison of the line slopes of different membranes can make plain their identity or their individuality.

In particular, in the validation effort, it is essential that the water/product ratio sanctioning the translation of the minimum water-wet integrity test value into the minimum product-wet value be valid (PDA Technical Report No. 26, pp. 16–17, 1998). The water-wet diffusive airflow line should be compared in its entirety with the product-wet curve. The two lines should be completely congruent. Single-point comparisons, however informative, simply will not suffice; the slope of the line is wanted. The same is true as regards comparing pre- and post-use integrity tests. Single-point comparisons have value, but are less revealing than multipoints.

Neither the bubble point test nor the single diffusive airflow determination by itself serves the purpose of integrity testing as well as do the multipoint analyses. However, once the slope of the product airflow line is at hand, single-point diffusive airflow testing is accepted in processing contexts. The likelihood in such cases of a dereliction between the 80% test point and the bubble point is judged acceptably reduced by the fuller characterization of the filter type, and by the safety margin added by the filter manufacturer. Interpretations can then be made on the basis of whether the single-point reading is on, over, or under the diffusive airflow line characteristic of the filter type.

When a drug product is used to wet the filter the bubble point will change from that of water; usually decreasing due to the reduction in the surface tensions of the liquids involved. Consequently, the 80% of bubble point level where water is the wetting fluid will likely represent a different level when product is used as the wetting liquid. However, there need be no problem in determining the product-specific bubble point. Its value may have undergone a shift from the water bubble point, merely because of the surface tension effect. A simple numerical displacement may not however, be assumed in deriving the single point diffusive air flow. The diffusive airflow displacement reflects not only the shift in bubble point occasioned by the liquid's surface tension, but it may be raised or lowered by the solubility differences of air (or nitrogen, where this is used) in the product as compared to that in water.

Pressure Hold/Decay Testing

The pressure hold test, also called the pressure decay or drop test, is still the most common test, where a manual test is performed. PDA Technical Report No. 26 describes a survey performed by the pharmaceutical industry in 1998. The report quotes that nearly 66% of filter users still perform the pressure hold test.

The pressure hold test is a variant of the diffusive airflow test. All is arranged as in the diffusion test except that when the stipulated applied pressure is reached, the pressure source is valved off. The decay of pressure within the holder is then observed over a predetermined time interval by using a precision pressure gauge or pressure transducer.

The decrease in pressure can come from two sources; the first being diffusive loss across the wetted filter. The upstream pressure in the holder decreases progressively all the while diffusion takes place through the wetted membrane. The second source of pressure decay could result from a leak in the filter system. Indeed, one virtue of the pressure hold test is that it does test the integrity of the plumbing: valves, gaskets, etc. In this function it serves as a leak tester. That is why it is often used as the initial integrity test. However, it can serve as an integrity test only because its correlation with the organism retention capabilities of the filter has been established by way of converting its test values to those of a multipoint diffusive airflow test. This enables its correlation with the organism retention values determined for the multipoint diffusion assay.

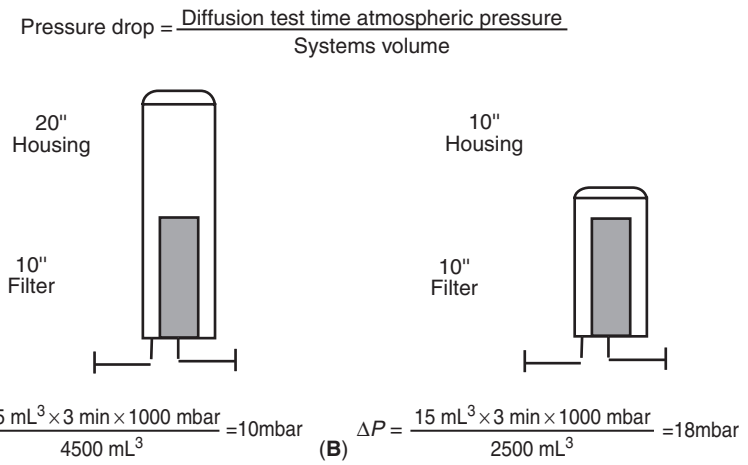

Pressure drop = Diffusion test time atmospheric pressure / Systems volume
20" Housing
10" Filter
10" Housing
10" Filter
(A) ΔP = 15 mL³ × 3 min × 1000 mbar / 4500 mL³ = 10mbar
(B) ΔP = 15 mL³ × 3 min × 1000 mbar / 2500 mL³ = 18mbar

FIGURE 11 Pressure drop variations due to different upstream volumes. *Source*: From Spanier, 1998.

Pressure Decay Basis

The pressure hold test measures the loss of air from within the filter housing. The perfect gas law governs the air loss measurement:

$$PV = nRT$$

where n is the moles of gas, R is the universal gas constant, T is the temperature, P is the gas pressure, V is the gas volume on the upstream side of the filter.

The decrease in gas pressure is influenced strongly by the upstream volume of the filter holder (Fig. 11). It depends on the particular holder-filter combination and the exact details of the filter and housing being used. Even the placement of the valve and any tubing volume must be considered, because this can affect the total volume within which the pressure decay takes place. Changing either the filter size or the housing necessitates a re-determination of the pressure hold value, for this may change the total volume surrounding the filter in its housing.

$$\text{Pressure drop} = \frac{\text{Diffusion vol} \times \text{Test time} \times \text{Atm pressure}}{\text{System volume}}$$

For Figure 11(A), the pressure drop is:

$$(\text{A})\Delta P = \frac{15\,\text{mL}^3 \times 3\,\text{min} \times 1000\,\text{mbar}}{4500\,\text{mL}^3} = 10\,\text{mbar}$$

For Figure 11(B), the pressure drop is:

$$(\text{B})\Delta\text{P} = \frac{15\,\text{mL}^3\text{x}\,3\,\text{min x}\,1000\,\text{mbar}}{2500\,\text{mL}^3} = 18\,\text{mbar}$$

The test is most often performed using automated testing equipment which, using appropriate (software) algorithms, converts pressure hold/decay data into diffusive flow data for which there are correlations to organism retention.

An advantage of the pressure hold/drop (or decay) test is that it determines the integrity of the entire filtration train including the filter. This test investigates the

possibilities for leaks or improper seals in the filtration system before the filtrative action is initiated. Performed using automated equipment connected upstream of the filter, it avoids risks to the membrane's asepsis and minimizes human subjectivity and operational errors. The pressure hold/drop test is described by PDA Technical Report No. 26, pp. 25–27; Trotter and Meltzer (1998); and in Jornitz and Meltzer (2001, pp. 426–34).

In the semiconductor and beverage industries, where integrity tests are used to signal conformation to the filter's specifications (rather than to reflect precise organism retention levels as required by FDA regulations in pharmaceutical processing), the pressure hold test can serve very well, provided that the measurement of the pressure decay is sensitive enough, and that a use history is developed for the given holder-filter combination employed.

In a personal communication, Hart (1998) suggested the use of a manual pressure hold test at the bubble point level, performing the test post-sterilization but before filtration. Due to the fact that the manual pressure hold test can be performed on the upstream side, this test can be performed post-sterilization without risks to asepsis. Steam sterilization is most stressful to the filter integrity; therefore, an integrity test should be performed after steaming. Raising the test pressure slowly to the minimum allowable bubble point limit given by the filter manufacturer, one only has to define the maximum allowable pressure decay level. This maximum pressure decay can be evaluated during the qualification stage of point tests at the same time. After filtration, the test is repeated and the before and after values are compared. This eliminates the risk of inaccuracies in determining the pressure drop via a pressure gauge, due to the higher test pressure level.

To be reflective of a filter's organism retention capabilities, the integrity test measurement, of pressure decay, for example, must be correlated to the measured property. Hango et al. (1989) describe the use of the pressure hold method as an integrity test under conditions of having the exact volume of the holder-filter combination and the relevant diffusive airflow defined by the filter supplier (Fig. 12). Under these circumstances, the pressure decay measured under pressure hold conditions (in the absence of plumbing leaks) can be correlated to ordinary diffusive airflow data that are in turn correlated with specific retention values.

Conversion of Pressure Decay into Diffusive Flow

The conversion of the pressure decay readings into diffusive flow values enables the transformation of the test into an integrity test qualified for sterilizing grade membrane

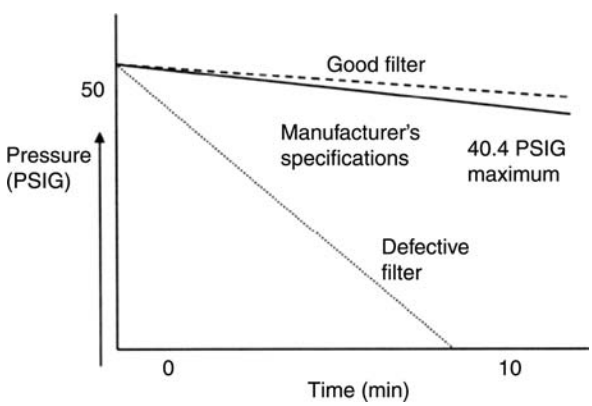


FIGURE 12 Filter integrity testing, pressure hold. *Source*: From Hango et al., 1989; courtesy of the Semiconductor Pure Water Conference.

filters. This can be done by calculating the diffusive flow of a filter element based on the pressure drop during test time t with a known upstream volume and reference pressure (1 atm or 1 bar) according to industrial standard DIN 58 356, part 2, and as shown in PDA Technical Report #26 Appendix D (Trotter and Meltzer, 1998).

Gas diffusion and the phenomenon of diffusive airflow are in accord with Fick's law,

$$N = \frac{DH}{L}(p_1 - p_2)\rho$$

where N is the permeation rate, moles of gas per unit time, *$(p_1 - p_2)$ is the* transmembrane pressure; H is the Henry's law solubility coefficient for the liquid-gas system, L is the thickness of the membrane, and ρ is the void volume of the membrane, usually around 0.8. The pore path being tortuous, L is longer than the membrane is thick; thus, the correction factor. The equation above demonstrates that the air diffusion involves the solubility coefficient. H in Henry's law addresses the solubility of test gases in various wetting fluids. Other factors that directly affect diffusive flow include differential pressure, the specific liquid/gas diffusion coefficient, the inverse relationship to membrane thickness, and, to a lesser degree, a correction factor for pore path tortuosity and other restrictions to fluid flow.

The significance of these factors dictates that the different wetting liquids and test gases will result in different diffusive flow values. Therefore, testing must be performed using the actual test gas and wetting liquid to show the direct and precise correlation of pressure decay with the calculated diffusive flow measurements.

The correlation to bacterial challenge tests and to the bacterial retentiveness of the filter must also be demonstrated. This is done by bacterial challenge testing on filters that were previously qualified by the diffusion test. Using filters with gradually increasing diffusional flows, one may elucidate at which value the filter will begin to pass test microbes. Using these diffusive flow data, a correlation to the calculated diffusive flow may be made and thus to the pressure drop/decay test.

$$P_1 V_1 \qquad \ln p$$

$$D = p_o t \qquad p_2 - \Delta p$$

where D is the diffusion in mL/min, V_1 is the upstream volume of filter system, p_1 is the starting test pressure, p_o is the atmospheric pressure of 1.0 bar, p_2 is the ending test pressure t = test time, Δp is the $p_1 - p_2$ in pressure units.

This formula compensates for the diffusive flow decrease as a result of the progressive pressure drop during the test time. This may become a significant factor when filter systems with high diffusive air/gas flow are encountered. This methodology is used by filter manufacturers to validate the pressure decay test and to qualify the various integrity test devices in performing these tests.

The translation of the pressure hold (decay) curve into the straight line of the diffusive airflow plot that relates to organism retention can be accomplished mathematically, as just shown. It is, however, performed automatically by the automated test machines designed for integrity testing. Moreover, these devices have the sensitivity essential for detecting the small pressure losses that may be involved.

Determinations of the pressure decays by means of the pressure gauges normally employed in the manual pressure hold tests are woefully inadequate. Conventional

pressure gauges lack the sensitivity required for the pressure hold test. If there are no sealing leaks, the pressure in a housing will decline about 1 psig over a 10 min period. After 20 min, it is to be hoped that such a drop can be noticed. In the event of system leaks, the pressure drops more precipitously. Small pressure drop readings may be of value in determining whether filter seating or housing O-ring leaks exist. Such readings are too insensitive to reveal much about the filter. Larger diameter gauges would be more useful but these and their periodic calibration are expensive.

Most of the automated integrity test devices use pressure transducers to measure the pressure drop in the upstream side of the housings. These measurements can be made with great accuracy. Pressure transducers have a sensitivity of ±1 mbar. That is why the automated instruments have the requisite detection sensitivity.

Pressure Hold Test: Pros and Cons

One advantage of the pressure hold test is that it is capable of revealing imperfections in the assembly and sealing of the housing and filters and disclosing flaws in the filters. This is particularly useful in initial integrity testing for the early disclosure of filter seating failures or housing sealing leaks, avoiding a profitless undertaking of the filtration.

A second advantage is that the pressure decay reading central to the pressure hold test can be made on the upstream side of the filter, on the housing itself, without compelling invasion of the downstream side of the system, even when done manually. Aseptic invasions and connections are common; they are made successfully every day. They do, however, pose a risk to the system and are best avoided where possible.

The use of automated test devices helps avoid downstream invasions during the performance of the more conventional integrity tests.

The major disadvantage of the pressure hold test is that it is strongly influenced by temperature, to an even greater extent than the diffusive airflow method. In the former case, temperature is a factor in the perfect gas law. In the latter, the solubility and diffusivity of the gas, as well as the volume it occupies, are influenced by temperature.

INTEGRITY TEST SCENARIOS

Integrity Test Preferences

Curiously, preferences for one particular test or another have been occasioned by the commercial rivalry that seems normal to competitive enterprises. Perhaps pride of innovation or of technical improvement motivates some to champion a given test. A reluctance to acknowledge a competitor's accomplishments may direct or misdirect the views of others. Then too there are always the possibilities of straightforward differences in evaluations and opinions.

In certain applications each of the integrity tests will serve equally well. The analyst may exercise his preference. There are, however, situations wherein the area of the filter, its EFA, compels the use of a particular test. Small filters, such as disc filters up to 296 mm in diameter, cannot produce diffusive airflows large enough to yield reliable results. The insufficiency can be overcome by the use of higher differential pressures, or by employing more sensitive measuring devices when available. But the practical need is best supplied by bubble point testing. Filters of larger EFAs can produce diffusive airflows so large as to obscure the bubble point readings. Bubble point assays, on the other hand, are best performed on smaller size filters where the interference of simultaneous diffusive airflows

are minimized. Where both type tests are possible, perhaps their mutual reinforcing actions should be sought to diminish the uncertainties of each.

Detecting Incompatibilities

Gross incompatibilities between membranes and fluids may be easy to discern. More subtle effects can be judged by their influence on the filter's bubble point. Any indication that contact between the filter and fluid tends to enlarge the pores is clear evidence of incompatibility. However, the bubble points can disclose incompatibilities that affect only the largest pores. Changes in the smaller pores are not made evident. The diffusive airflow readings mirror the influences of all the pores (total porosity). Thus, the use of diffusive airflow measurements may indicate potential fluid/filter incompatibilities with greater sensitivity than do bubble point determinations.

The pore size alterations caused by heating stresses can likewise be detected, as also the kinetics of the process. However, in these activities it were best that the slopes of the plotted multipoint lines be compared, rather than those of the single-point rate Figure 13 illustrates the diffusive airflow analysis revealing flaws induced in a membrane subjected to the stresses of repeated steam sterilization cycles. In diffusive flow measurements, what is sought is the point of departure from the constant slope of the diffusive airflow line. Automated test equipment measures the gas diffused over a period of, say, five minutes. If the maximum flow rate is not exceeded, the pressure is raised appropriately.

Integrity Testing Multi-Round Assemblies

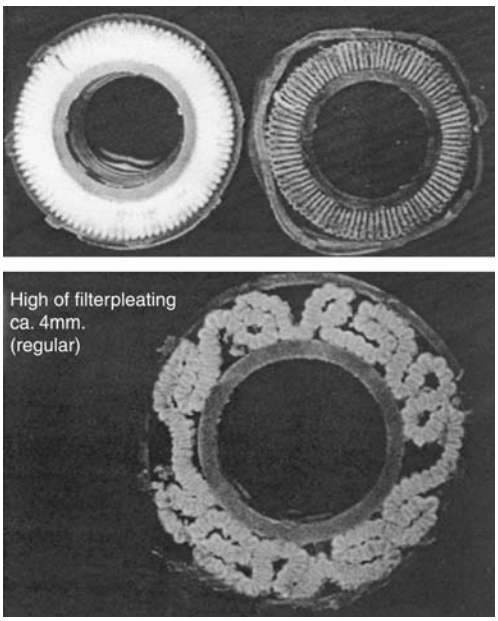
A number of problems require resolution in the search for methods to determine the integrity testing of multiple cartridges in multi-round housings. One wishes to eschew the testing of the individual cartridges in the laboratory followed by their insertion into the housing under aseptic conditions. What is required is the clear determination of the assembled cartridges' bubble point. It is assumed that a single failed cartridge could be detected, and that this would suffice to impugn the integrity of the entire assemblage. Presumably, one might by selective assumptions calculate the chances for one failed filter not to affect the function of the others. An encouraging positive conclusion might then lead to calculations involving two faulty filters, etc.

There would still remain the problem of the combined diffusive flows from the assembled cartridges being large enough to make impossible the recognition of the filter combination's bubble point. Clearly, the larger the number of cartridges involved, their lengths, porosities and the differential pressures involved would have to be balanced against the airflows resulting from the single or multiple flaws.

Even were it possible to hypothesize a realistic situation involving these several factors, the arbitrary, if necessary, assumptions would likely not represent the complex combination of flows that the situation actually offers.

As helpful as a solution to the stated problem would be, it is not likely that it will emerge from an hypothesized set of conditions even were it to involve two filters at the most. Where two filters are involved, the balancing of the combined bulk airflows against the combined diffusive flows could be achieved, but at the cost of assumptions that may or may not be realistic. The difference between the bulk flows and the diffusive flows would have to be so large as not to confuse the former by the latter. There is no alternative otherwise to aseptically testing, and housing the filters individually.

(**A**)



(**B**)

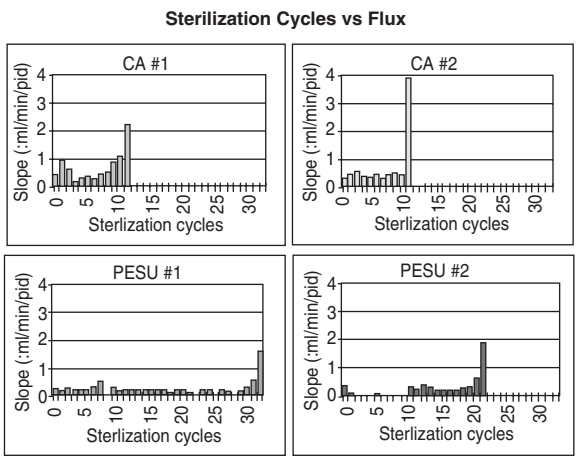


FIGURE 13 (**A**) Fitter damage due to excessive steaming. (**B**) Sterilization cycles vs. flux.

Repetitive-Filter Effects

The pore is modeled as being a convoluted, irregular capillary. Because of its tortuosity, it is necessarily longer than the depth of the filter. Its restrictive dimensions may occur anywhere along its length. This assumption explains certain observations regarding repetitive filters. It would be expected that lengthening a pore might proportionally increase the pressure drop, i.e., decrease the differential pressure across its span. This should reduce its flow rate; promote its adsorptive particle-trapping abilities; but probably not alter its throughput. The actual situation wherein membranes are superimposed in intimate contact on one another in effect does prolong the pore length. The expected consequences follow.

The congruent positioning of the filter layers will cause a homogenization of the pore size distribution. There will result fewer large size rated pores because pores of large pore size ratings in either filter will likely juxtapose those with those of smaller ratings in

the other because there are many more of the smaller. The result will be a diminution in the overall pore size. This should assist sieve retentions. Moreover, the pore paths are doubled in their lengths, as are also their surfaces. This encourages adsorptive sequestrations. The filter efficiency is enhanced.

Where use is made of two membranes that are separated from one another, a different result is obtained. Between the two membranes there exists a space wherein the fluid exiting the first membrane forms a pool from which it is hydrodynamically directed to the larger pores of the second membrane. The hydrodynamic flows preferentially convey particles to the more open pores. This detracts from the narrowing of the pore size distribution's doubling of the single filter's pore length (Fig. 14) (Reti et al., 1979).

To repeat, the LRVs of individual filters are not additive in their combinations. The exact sum depends upon the probabilities of certain size organisms meeting an appropriately sized pore within the pore size distribution. Whether by encounter with a larger pore, or with a flaw, the likelihood is that the organisms penetrating the upstream filter will be the smaller of the organisms present in the feed stream. This will decrease the size distribution of the organisms downstream of the filter in the direction of overall smaller sizes. This, in turn, makes more likely a lower LRV for the downstream filter (Reti et al., 1979; Sundaram et al., 2001, Part 1; Trotter et al., 2002).

Redundant Filtration

This term is often used to designate a repetitive or two filter arrangement wherein the two filters, each in its own cartridge, are housed separately. The term "redundant" often conveys a pejorative cast to the usage as being wasteful or unnecessary. Its intended meaning in filtration is to imply "enough and to spare." The first filter, as is common to repetitive filters, is expected to fulfill the retention requirement. The second filter serves as insurance. As already observed, the LRVs of repetitive filters are not twice the value of a separate filter.

Economics of Redundant Filtration

Redundant filtration incurs the cost of two housings, but the separate housings permit the integrity testing of each filter. Within a single housing the upstream testing of the

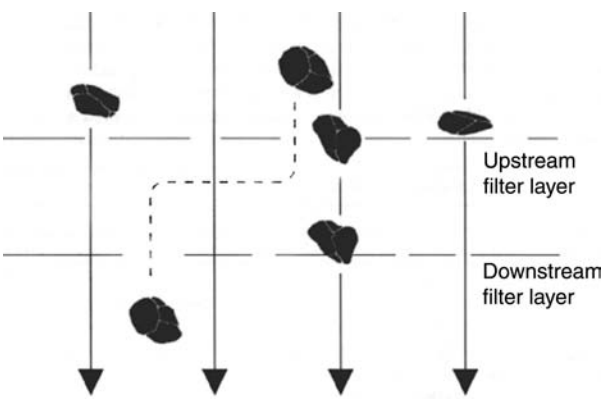


FIGURE 14 Effect of separated repetitive layers: retention and integrity testing.

downstream filter is manageable, but will require the isolation of the upstream filter; not a simple undertaking.

The economics of using redundant filters depends upon the value of the loss of a batches of drug product, the frequency of batch failures, the total cost of the redundant filters to prevent the batch losses, and the expense in downtime and effort needed for filter replacement. The sum total of these costs is to be offset by the value of the saved drug batches. The likelihood of savings resulting from the reliance on redundant filters is more likely positive when the production of the more costly biotech drugs is involved.

Regulatory Recommendations

As regards the EU and FDA, both the Guideline CPMP, April 1996, and EC Annex 1, as well as the FDA's Aseptic Guide (2004) recommend the use of redundant 0.2-/0.22-μm-rated membranes. FDA's new aseptic guideline states, "use of redundant filtration should be considered in many cases." The placement of the filters is to be "as close as possible" (or practical) to the filling needles. The usual location is indeed just before the filling needles or before the reservoir that feeds them. In practice the "recommendations" of a double filter arrangement tend to be enforced as if they were law. In Europe, this usage is becoming the common practice.

HYDROPHILIC FILTER INTEGRITY TEST CONSIDERATIONS

The Wetting of Surfaces

The wetting action of a solid by a liquid is illustrative of the bonding interaction between the molecules of the liquid and those of the solid surface. Bubble points reflect the cohesive force of such a wetting action between a specific liquid, for example, water, in contact with a specific solid surface, as of a capillary or pore wall. Given the pairing of specific molecular entities, this particular cohesive force is unique. Its value depends upon the specific liquid and solid molecules involved.

The strength of the adhesive forces are expressions of the surface free energies involved. The critical surface tension of a solid is identified by that of a liquid whose intimacy of wetting results in its spreading over the solid surface. The greater the surface tension similarity between liquid and solid, the more avid the molecular interaction (hydrophilic), and the more complete the wetting. Conversely, the more unalike the solid's surface tension is from that of water's 72 dyn/cm, the more difficult it will be to obtain water-wetting. The membranes employed in filtering aqueous compositions need to be hydrophilic. Their critical surface tensions should approach water's high value of 72 dyn/cm. Most of the polymers used in membrane construction meet this criterion, although some fall into a range of borderline "wettability" and may water-wet with reluctance. Assistance may be required to completely water-wet such filters. The surfaces of the smaller pores are more difficult to wet because their narrow lumens are difficult to intrude.

The failure to obtain complete and total wetting will leave some pores filled with air not displaced by water in the wetting exercise. This will lead to error in measuring filter properties associated with flows and flow rates, including bubble points.

Complete wettability results in the liquid's spreading itself over the solid's surface. This occurs when the adhesion between the solid and liquid molecules is greater than the cohesion between the like molecules of the liquid. Less avid wetting tends towards the beading of the liquid in droplet form upon the solid. The hierarchy of wetting extends from the complete spreading of the liquid over the solid, to its appearance as spherical beads on

the solid surface. In less than perfect wetting, an angle is formed at the contact site of liquid and solid. It becomes more pronounced, the greater the imperfection of the wetting. The more spherical the liquid drop, the greater the angular space between the flat surface of the solid and the upward curvature at the bottom of the spherical liquid bead (Fig. 15). Called the contact angle or the angle of wetting, it is symbolized by the Greek letter theta, Θ.

The degree of wetting imperfection reflects the differences in the cohesive energy densities (CED) more properly their solubility parameters, the square root values of the CEDs, of the liquid and solid molecules that determine their structural similarities. The less-alike their molecular architecture, the less their mutual attraction, and the greater their free surface energies. These find expression in surface tension values. Called the critical surface tension, the surface tension of the solid is revealed by that of the liquid that fully wets it. The significance of the critical surface tension will be made more evident in the section Ensuring Complete Wetting.

Water-Wetting the Filters

The tests or test suited to the examination of hydrophilic filters require a *complete* wetting out of the filter. The pores most difficult for water to intrude and to wet are most likely the narrowest, or as commonly labeled, the smallest. The air content is replaced by water which in combination with a pressurized gas enables measurements of the rate at which the gas diffuses through the wetted filter. The measured airflow quantities translate into assays of filter integrity. Ensuring constant temperature and complete wetting are basic requirements.

The incomplete wetting out of the filter is the most frequent cause of integrity test failures. The diffusive airflow rate is expected to be proportional to the differential pressure. When water does not fill all the pores, the unfilled pores, not blocked by liquid, permit premature bulk air flows. The flow, enhanced beyond the normal for its pressure differential level, is interpreted as indicating a flawed filter.

Solid surfaces, such as pore walls, are wetted by liquids having surface tensions similar to their own. Water has the high surface tension of 72 dyn/cm. To wet filters that are less hydrophilic requires liquids of lower surface tensions, closer to the critical surface tensions of the less hydrophilic filter polymers. The critical surface tension of polyethylene is 32 dynes/cm; of polypropylene 29.5; of polyvinylidene fluoride (PVDF) 25; and of polytetrafluoroethylene (PTFE) 18 dyn/cm. Aqueous alcoholic solutions exhibit surface tensions lower than that of water. Their numbers are those of water (high), moderated by admixture with the lower numbers of organic alcohols. For example:

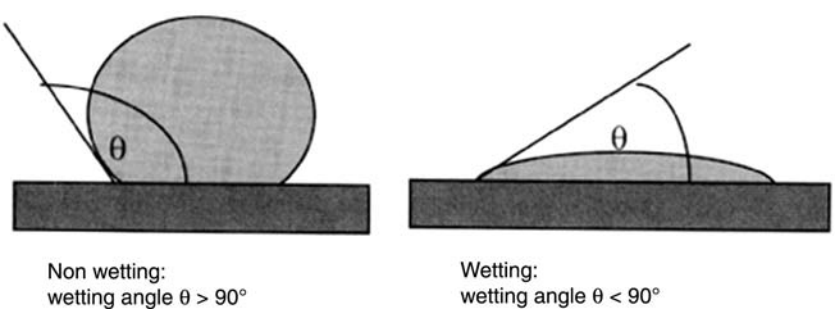


FIGURE 15 Liquid surface interaction at different wetting properties.

aqueous alcoholic solutions of ~60–70% v/v, methanol, ethanol, isopropanol, or (rarely) 25% v/v tertiary butanol are employed to wet completely filters that wet reluctantly with water alone. The subsequent elimination of the alcohol from the system is accomplished by a thorough water flush performed before the aqueous alcoholic solution evaporates from the membrane surfaces and leaves them dry.

Product as the Wetting Liquid

Often, efforts are made to flush the filter with water before running the final integrity test, so that pre-and post-filtration bubble point tests using water are obtained for comparison. However, even copious water flushing may not restore the water bubble point. For example, it was reported that nylon membranes became fouled by proteins in an albumin filtration process that often enough the filters were not wetted by water and a false negative results was obtained. Same was found with products containing Tween. Even after large water flush volumes, the surface tension reducing properties were seen. In such cases, pre- and post-filtration comparisons may usefully be performed using product as wetting agent for the filters. The displacements in bubble values being ascribed to unknown wetting effects, but largely to the influences of the surface tension values of the product, are assumed not to reflect on the organism removal capabilities of the membrane.

More often, post-filtration integrity testing is performed by using the product filtered as the wetting agent, due to the fact that its removal by water flushing may require excessive amounts of the liquid water. Certainly the contact between certain membranes and various pharmaceutical preparations can produce depressed bubble points compared with the values for water (Table 1). The depressed bubble point can be restored, more or less, but mostly less, by copious washing of the filter with water, depending on the filter material and/or product ingredients. Some subtle wetting effects, adsorption or fouling involving product ingredients may be at work here whose surface physics is not comprehended. In addition, the surface tension differences between the product and water are contributory to the anomaly.

Regulatory authorities also advocate performing bacteria challenge tests with the actual product under process conditions. Such challenge tests, involving also viability

TABLE 1 Bubble Point Values for Different Wetting Agents Using Cellulose Acetate 0.2 μm

Product	Bubble point value (bar)
Water	3.20
Mineral oil	1.24
White petrolatum	1.45
Vitamin B complex in oil	2.48
Procainamide HCl	2.76
Oxytetracyline in PEG base	1.72
Vitamin in aqueous vehicle	2.07
Vitamin in aqueous vehicle	2.69
Iron dextran	2.83
Vitamin E in oil base	1.66
Solution preserved with benzyl alcohol	2.14
Diazepam in glycol base	1.93
Digoxin in glycol base	2.14

testing, confirm the filter's retentivity. Moreover, they reveal any negative influences of the product towards the challenge organism (Mittelman et al., 1998).

Parker (1986) determined the acceptable minimum bubble point for a given type of filter using product as wetting medium in accordance with the formula

$$P_p = \frac{P_o}{P_w} P_m$$

where P_p is the minimum acceptable product bubble point, P_o is the observed bubble point using product, P_w is the average of the water bubble points observed for samples of the filters (commonly 3 filters from 3 different batches), and P_m is the filter manufacturer's stated minimum allowable bubble point. Enough filters or filter devices are secured from each lot of the subject filter type to yield an acceptable average value. Testing is performed for each product being filtered using 47-mm disk filters or small-scale pleated filter devices.

Desaulnier and Fey (1990) confirmed Parker's findings. Parker et al. describe the exact protocols by means of which the product bubble point may be determined. The latter authors also describe an apparatus suitable for the purpose.

More recently, the PDA Technical Report No. 26 describes such product integrity evaluation thoroughly. The formula, in itself the same as described by Parker, is, with the recommended test procedure, described, in addition to a statistical approach, using Student's *t*-distribution to obtain a "corrected product-wetted bubble point." Due to the fact that the evaluation of the product wet bubble point can include a high variability, the statistical evaluation is required to take account of such variabilities. Following equation is used:

$$\mathrm{CPBP} = \mathrm{PBP}_{\min} - (t_{\alpha \mathrm{df}})\mathrm{S}$$

where CPBP is the corrected product-wetted bubble point (bubble point limit used for production filters), $\mathrm{PBP}_{\min}$ is the minimum product-wetted bubble point established by multiple integrity tests, using Parker's formula, $t_{\alpha \mathrm{df}}$ is the t value from Student's table associated with a confidence level α and degrees of freedom and s the standard deviation.

The report also quotes an example in Appendix E, using a one sided Student's test with a 95% confidence level, α. Seven bubble point tests were performed, the individual values are stated in Table 2. The average water-wetted and product-wetted values were calculated by adding the measured values for water and dividing it by 7 (same for the product values).

TABLE 2 Example of Measured Product-Wetted and Water-Wetted Filters and the Resulting Average Values and Correction Ratio

Water-wetted bubble point (psi)	Product-wetted bubble point (psi)	Ratio
53.1	43.7	
52.8	43.6	
53.1	43.5	
52.1	42.4	
52.1	42.8	
55.3	46.1	
55.4	46.5	
53.4 (average value)	44.1 (average value)	0.826

Source: Courtesy of PDA, Bethesda, Maryland.

A correction ratio of 0.826 is calculated by dividing the product-wetted average bubble point value (PBP_{avg}) 44.1 psi by the water-wetted average bubble point value (WBP_{avg}) 53.4 psi.

The minimum allowable water-wetted bubble point (WBP_{min}) is in this example 53.7 psi. These values are usually given by the filter manufacturers and are correlated to bacteria challenge tests. The product-wetted bubble point value (P_{min}), is now calculated by multiplying the correction ratio, 0.826, with the minimum allowable water-wetted bubble point value, (WBP_{min}) 53.7 psi. Therefore, the product-wetted Bubble Point value (PBP_{min}) is 44.4 psi (0.826×53.7 psi).

The standard deviation, *s*, for the product-wetted bubble points values in column 2 is 1.587 psi. The t-value at 95% confidence level, for $n - 1$ degrees freedom (in this example $n = 7$) is $t_{.95,6} = 1.943$. Multiply *t* by the standard deviation, *s*, to obtain the correction factor of 3.084 psi (1.943×1.587 psi).

Now the corrected product-wetted bubble point, using $CPBP = PBP_{min} \times (t_{\alpha df})s$, can be calculated. $CPBP = 44.4 \text{ psi} \times 3.084 \text{ psi} = 41.3$ psi. This value admits a certain variability within the evaluation without compromising the integrity test procedure and levels.

Usually the evaluation of the so-called product integrity test values requires three filter membranes or devices of three different lots, that is, nine tests in total. At one point it was recommended that one of these filter lots must be close to the minimum allowable water bubble point value given by the filter manufacturer to ensure retentive capability at the established limit values. This factor is now included within the corrected product-wetted bubble point value evaluation.

Wetting and Temperature Requirements

The water flow is always from the outside to the inside; through the outer cage of the cartridge, through the filter layers, to its exit by way of the inner core. A thorough wetting out of the filter is needed to prepare it for integrity testing. It needs to be completely wetted, as well, for steam sterilization, and for its application of filtering aqueous preparations.

The majority of integrity test failures stem from the incomplete wetting out of the filter. There is uniform standard wetting procedure. It seems reasonable that the filter manufacturer's protocol should be followed. Some recommend specific flush procedures suitable for their membrane polymers and filter configurations. Some filter purveyors provide pertinent advice by way of troubleshooting guides. Figure 16 shows such information presented as a wetting-tree recommended by one filter manufacturer.

Recalcitrant cases may benefit from the use of hot water (100–200˚F; 38–84˚C). However, the influences of the higher temperature have then to be avoided, especially in integrity testing, by flushing the filter system with cold water. Alternatively, the system is allowed to cool down before the integrity test is performed.

Filter Failure Inquiries

The second most likely cause of failure is damage to the filter. Due to the delicacy of the membrane the damage is seldom subtle, and the failure is abrupt. Integrity test failures resulting from chemical incompatibilities can become progressively evident, depending on the severity of the chemical mismatch. As it involves filters, it is usually in the nature of a plasticization. It may be rate controlled by the diffusion of the liquid into the solid. Therefore, prior to testing, the contact time between filter and fluid should equal the processing duration.

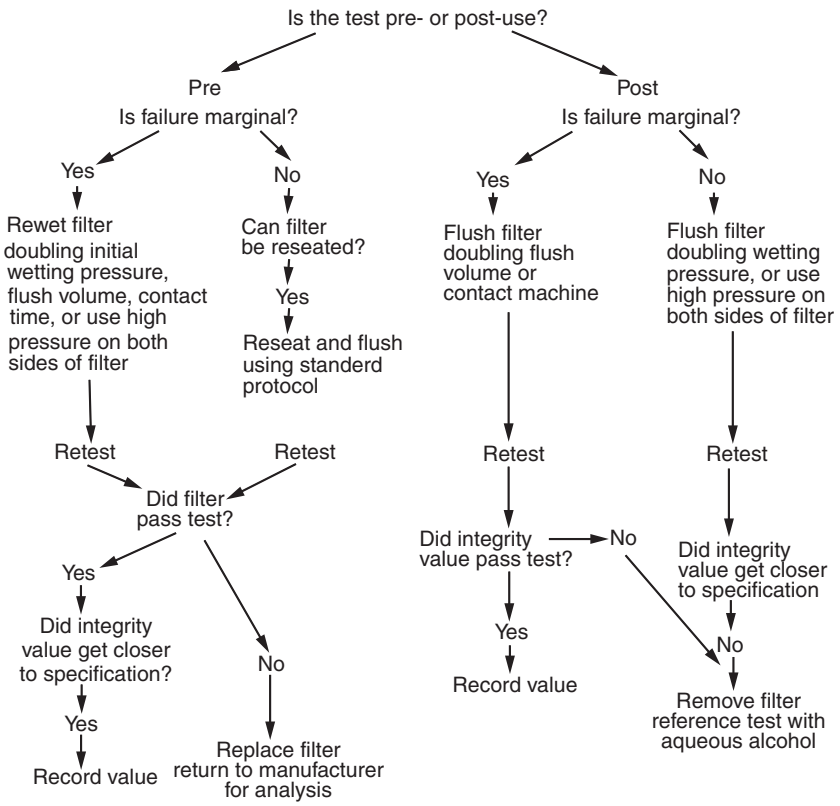


FIGURE 16 Wetting guide.

An early confirmation of the filter's identity can be obtained. The compatibility of the filter and the drug preparation relative to organism retentions, such as pore size alterations, can be ascertained in an exploratory study by comparing the integrity test values before and after exposures of the filter to the drug product for a period of time equal to at least the duration of the processing filtration. Pore size modifications caused by steam sanitizations may also be assessed in an exploratory study after a steam/filter contact time equal to that of the processing step itself. It is necessary to allow the steamed assembly to cool (or to be cooled) to the proper temperature before the testing begins; the test results being temperature sensitive.

Gas Permeation of a Wetted Filter

Let us consider the origins of the gas flows generated in arriving at a filter's bubble point. Most commonly, air pressure is applied to the wetted filter, pores are filled with water. Nitrogen gas is to be preferred because of its lower moisture uptake (Hofmann, 1984).[c]

[c] Hofmann (1984) states that compressed air has a greater water holding capacity than nitrogen. Its use, he believes, would excessively dry the membrane by affecting the distribution of the water within the membrane pores. This would add error to the test results. However, this has not materialized significantly in practice.

Carbon dioxide is not suitable because its high solubility in water produces a diffusive air flow rate too high to be useful in the measurements.

Under the applied pressure, the gas molecules dissolve on the higher-pressured upside of the filter in accord with Henry's Law which states that a gas is soluble in proportion to its partial pressure above the solution. The dissolved gas exists in a true state of solution; its individual molecules disperse among the water molecules. From their high partial pressure at the point of their introduction upstream of the filter, the dissolved gas molecules diffuse to the lower partial pressure areas downstream of the filter, to equilibrate their concentration as dictated by solution physics. The differential pressure across the filter compels the attainment of this equilibration.

The elevated partial pressure at the inlet site undergoes a successive reduction to its lowest point at the atmospheric pressure side of the filter. The ensuing progressive decrease in the solubility of the gas reflects its decreasing partial pressure. This is sufficiently reduced at its exit from the filter for the gas to be less soluble, and to emerge from solution. It is seen as a stream of bubbles, measurable by their rate of collection as a gas, or as volume displacements of water from a burette.

Constant Temperature

To accommodate the requirement for constant temperature conditions in the pressure hold test, the appropriate automated testers include equipment to determine whether the temperature is within the acceptable range. Otherwise, temperature deviation can be seen on the graph printed by the machine. If the temperature is not constant enough, the machine displays a warning.

Figure 17 shows what can happen when a higher temperature source heats up the upstream volume of the filter's housing. Even a brief touch of the hand to the housing immediately shows on the printed graph (Hofmann, 1984). Such influences may not be detected by a manual test. This poses a risk. An automated test machine is able to evaluate the status regarding temperature.

Temperature effects can be minimized by using a larger upstream volume, which may compensate for temperature drift. Nevertheless, the accuracy of the test would suffer with a larger upstream volume and taking a manual pressure drop reading may prove difficulty.

Another possible disadvantage could be the need for an accurate measurement of the upstream volume for the determination of the pressure decay level. Measuring the upstream volume manually, when performing the test manually, will give highly inaccurate results. This inaccuracy will be carried over to the actual measurement of the pressure drop

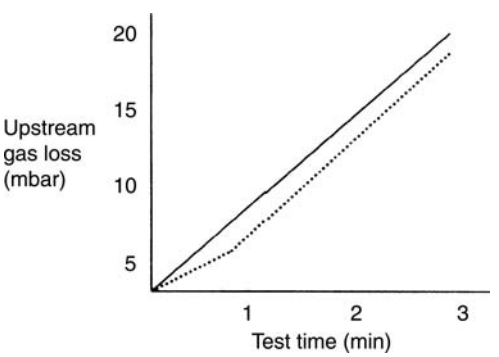


FIGURE 17 Temperature effect on diffusive flow. *Source*: From Hofmann, 1984.

(Trotter and Meltzer, 1998). It is, therefore, essential that the upstream volume be known with an exactness in order for the pertinent calculations to reveal their true significance.

Filter Area Effects

It is, for instance, not possible to completely separate the diffusive airflow component from the bulk airflow at the bubble point. Therefore, the bubble point examination is more suited to the testing of filters whose areas are small enough not to incur large diffusive flows that would overwhelm the detection of bubble points. The filter being assayed should, however, be large enough to produce a diffusive flow large enough to be measured with accuracy.

Whether the diffusive airflow given by a filter interferes with the accurate determinations of the filter's bubble point depends upon the EFA of the filter and the length of time necessary to arrive at the bubble point. During the progressive pressure increases on the way to the bubble point, diffusive airflow will be taking place. If, in the interval over which the bubble point is reached, enough air becomes diffused to substantially match the bulk airflow at the bubble point, the latter determination becomes uncertain. This effect becomes more noticeable when liquids of lower surface tension are involved, such as solvent-water mixtures.

Generally, the diffusive airflows from 10 inch cartridges begin significantly to interfere with the bubble point as it is commonly performed. However, even multiple 10 inch cartridges can and are successfully bubble pointed provided that the pressure is rapidly brought to just below the presumed bubble point, and then carefully, but not leisurely, raised to the actual bubble point. There are limits even to this helpful technique. At some point the filter area is large enough to provide diffusive airflows that will interfere with the bubble point regardless of how expeditiously performed. The relevant area will differ for different filters, and, therefore, cannot be defined with exactness.

Consider a 10-inch cartridge with a diffusive airflow of 15 ml/min. At its bubble point it will have a certain bulk airflow, far in excess of 15 ml/min.; perhaps about 540 ml/min. Three such cartridges joined into one 30 inch assembly will have a diffusive airflow of 45 ml/min but the same bulk airflow at the bubble point, namely, about 540 ml/min if only a single section were involved. (If the bubble point is simultaneously reached at more than a single cartridge, the air flow will be proportionately greater). The bubble point is identifiable; 540 ml/min diluted by 45 ml/min.

An assembly of three individual 30-inch cartridges could have a diffusive airflow of about 136 ml/min and the viscous airflow at the bubble point of 540 ml/min or so. The difference between 135 and 540 ml/min is large enough not to confuse the two different airflows provided the time to reach the bubble point is minimized. If, however, it takes 4 or 5 min to reach the bubble point, then the diffusive airflow will not be distinguishable from the bubble point because the total diffusive airflow and the bulk airflow (at the bubble point) will be equal. The latter will be confused by the former. If an assembly of twelve 30-inch cartridges is involved, its diffusive airflow will be 540 ml/min. No matter how fast the bubble point is determined, it will be interfered with by the diffusive air flow emanating from the filter arrangement.

INTEGRITY TESTING HYDROPHOBIC FILTERS

The hydrophobic membranes find application as air filters, generally as vent filters. These protect the contents of the tanks or other containers against contamination by the air that

enters or leaves them as occasioned by the addition or withdrawal of their stored liquids. The concern is with the possibility of water finding lodgment in the membranes as a result of splashing, foaming, or even by the condensation of water vapor. Liquid water would possibly impede air passage, conceivably leading to tank collapse upon out-pumping; moreover, water would encourage microbial growth. Contamination therewith is strongly to be avoided. The prescription against such an occurrence is to deny the presence of moisture. This can be done for hydrophilic filters using electrically heated or steam-jacketed filter housings. The temperature is raised above the dew point of the ambient air. Usually, steam at 1/3 bar (5 psi) pressure is enough. Unfortunately, the long-term passage of air through such heated arrangements will cause the oxidative degradation of certain filter cartridge components, such as polypropylene, and may also oxidatively degrade the microporous membrane itself, depending upon its composition. Fluorinated polymers are relatively immune to oxidative alteration; PTFE extremely so, PVDF somewhat less so. That is why these polymers are so widely used in air filtration contexts. The greater hydrophobicity of PTFE offers substantial advantages.

Pore Blockage by Water Condensation

Mechanical considerations also favor the use of hydrophobic membranes for air filtration. Water intruding on air or vent filters, whether by splashing or condensation, can be removed from hydrophobic structures by the imposition of a pressure sufficient to overcome its bulk inertia. In the case of hydrophilic air (vent) filters, however, higher applied pressures are required to outweigh the adsorptive forces holding the water within the pores. Pressures in excess of the bubble point are needed to vacate even the largest pores, and pores of smaller diameters will require even more elevated pressures. The result is that water, splashed or condensed, within the hydrophilic membrane can cause serious blockages of pore passageways to the detriment of airflow. In the case of vent filter, tank implosions are risked when, under such conditions, liquid is pumped from storage tanks not protected by rupture disks or blowout patches. It is therefore considered advantageous to avoid the possibilities of such incidents through the use of hydrophobic air filters.

Testing with Lower Surface Tension Liquids

As stated above, hydrophobic materials are generally characterized by low critical surface tensions. They, thus, resist wetting by water with its high, 72 dyn/cm^2 surface tension. However, aqueous alcoholic solutions of the compositions given above are of lower surface tension values and serve as wetting fluids adequate for bubble pointing fluoropolymeric membranes. This technique requires, however, the subsequent disposition of the wetting liquid. Such can be accomplished, but by rather complex plumbing arrangements, or by risking asepsis downstream of the filter.

Water Penetration Test

It has long been understood that water can be forced through hydrophobic filters under extreme pressures. The pressure necessary for this action relates inversely to the diameters of the pores. This situation involves the same wetting considerations that govern the time-honored mercury porosimetry procedure of probing the diameters of microporous membranes. Non-wetting mercury permeates glass capillaries or other porous materials that are too different in their surface tension characteristics to be wetted by the liquid metal. However, defining the relationship between pore diameter and the pressure required to force water through a hydrophobic filters does not in itself constitute

an integrity test. For that conjunction, a documented correlation must be demonstrated to exist between the intrusion pressure and acceptable levels of organism retention by the filter. Such a relationship has been reported, thereby establishing the water permeation test as an integrity test applicable to the integrity characterizations of hydrophobic filters.

However, the vent filter's pores having been permeated by water must then be made rid of the liquid by a drying operation. This requirement was time consuming and costly in terms of heat. It was found that a better test to the same purpose resulted from measuring the intrusion pressure rather than the penetration pressure involving the hydrophobic filter material and the liquid water.

Water Intrusion Pressure

The water intrusion test measures the decay rate of a pressure level imposed upon a hydrophobic filter enveloped by water. By means of an automated integrity tester, a particular decay level is identified as the point at which water enters the largest pores of the filter.

Hydrophilic Surface Deposits

The water intrusion test depends upon and measures the hydrophobicity of the filter. Only the experimentally demonstrated correlation of its values with the entrance of water into the hydrophobic pore structure establishes it as an integrity test. But the basic measurement of filter hydrophobicity is itself an inherent requirement in its pertinence to air filter reuse. Consider an integral air filter that contains hydrophilic accretions or deposits upon its surface. Their presence may encourage microbial growth and, ultimately, organism penetration. Solely ensuring air filter integrity, however essential, is not enough in air filter usage. The filter's full hydrophobicity must also be assessed to ensure freedom from compromising hydrophilic impurities.

Long-Term Air Filter Applications

As previously stated, in the usual air filtration or vent filter applications, the filter is so little exhausted by the depositions it collects that its reuse is compelled for economic reasons; hence the need periodically to repeat its integrity verification before and after each individual use, ideally without removing it from its installation. The filter may remain integral, but if it collects, as it well may, deposits of hydrophilic matter, then as localized and limited as these may be, they can serve as loci for organism growth and penetration, defeating the very purpose intended by the use of a hydrophobic filter. If the intention of the integrity test in air filtration applications is solely to assess filter integrity, then the water intrusion test may, on occasion, mislead and be responsible for the discard of integral filters. If, however, the purpose of examining the filter is to gauge its suitability as a longer-term air filter, as in fermenter operations for example, then the water intrusion test is sovereign for the purpose because it simultaneously measures the integrity and the hydrophobicity of the subject filter. Both of these properties are required in a filter dedicated to longer-term air filtration applications.

When the water intrusion test indicates an integral filter, it may be used (or reused) with confidence, even in long-term air applications. Failure to pass the water intrusion test signals a need for further filter assessment. A bubble-point test subsequently performed and passed successfully may indicate that the filter is not sufficiently free of deposits to permit its safe reuse, albeit integral. Then if considered desirable, a suitable filter-refurbishing effort may be undertaken.

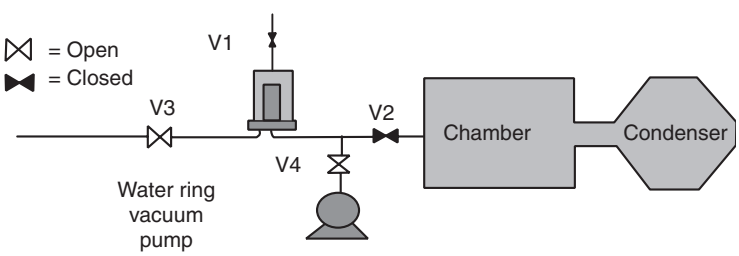
In passing it can be remarked that hydrophobic membranes, widely employed as vent filters, are not wetted by water, and that a certain pressure is, therefore, required to force the water into them. This pressure, called the water penetration pressure (WPP), is different for different filter membrane polymers. For example, the WPP for PTFE is around 4.5 bar (65 psi), and that for PVDF is around 2.8 bar (41 psi). Water penetration pressure depends also on the pore size of the individual filter membrane.

The upstream side of the hydrophobic filter cartridge housing is flooded with water. Air or nitrogen gas is then forced into the upstream side of the filter housing above the water level to a defined test pressure by way of an automated integrity tester. A period of pressure stabilization is allowed over a time frame recommended by the filter manufacturer. During this interval the cartridge pleats adjust their positions under the imposed pressure. After the pressure drop thus occasioned stabilizes, the test time starts, and any further pressure drop in the upstream pressurized gas volume, as measured by the automated tester, signifies the beginning of water intrusion into the largest (hydrophobic) pores, water being incompressible. The automated integrity tester is sensitive enough to detect the pressure drop. This measured pressure drop is converted into a measured intrusion value, which is compared to a set of intrusion limit, which has been correlated to the bacterial challenge test. This correlation has been demonstrated between the observed pressure drop and the organism retention characteristic of the hydrophobic membrane (Tarry et al., 1993; Dosmar et al., 1993; Meltzer et al., 1994; Jornitz et al., 1994; Tingley et al., 1995). This empirically established correlation serves as the validating authority for the water intrusion test.

Drying of Filters

When the test is over, the water is drained from the upstream side of the filter housing and the filtration can be started. In terms of a freeze dryer vent filter, one has to dry the filter after the WIT before the freeze drying can be started, because of the need to avoid residual water.

A water-intruded filter can be dried by pulling a vacuum and creating airflow through the filter. This methodology is described by Bardat et al. (1996, 1997). The test setup is shown in Figure 18. The procedure has the advantage of being fast-working and efficient. Drying such filters by using heat-jacketed filter housings or pipework and running the warmed air through the filter will not have the same effect. Drying with a heat jacket will take more than 5 hr. By using this procedure, hydrophobic vent filters can be



time (min)	0	30	60
residual water after WIT	52,9 g	13,6 g	0,4 g

FIGURE 18 Lyophilizer vent filter drying after WIT. *Source*: From Bardat et al., 1997.

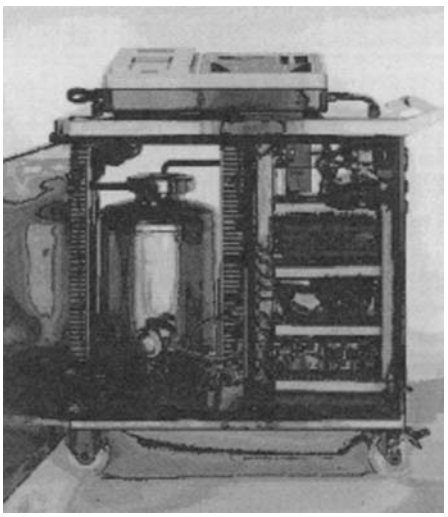


FIGURE 19 Automatic water intrusion test trolley. *Source*: Courtesy of Sartorius AG, 1995.

integrity tested in situ without the use of alcoholic solutions and the risk of downstream contamination.

To simplify the test for users, filter manufacturers offer fully automatic test units (Fig. 19) that, once connected to the appliances, make it possible to run the test without user intervention. This saves time and increases the reliability of the test.

The water intrusion test, like other integrity tests, is sensitive to temperature and to the water purity in terms of surface tension. It is, however, less sensitive to temperature variations than the bubble point and diffusive airflow measurements because of the large thermal mass represented by the water in the housing. Nevertheless, when a hand is placed on the housing wall containing the air volume above the housing, a temperature effect can be detected. The basis for this integrity test has already been discussed.

Correlations have been established between the organism retentions exhibited by a filter and the rates of pressure drop from a given pressure level as caused by water intruding into a hydrophobic filter. The greater the rate of pressure drop, the greater the extent of water intrusion into the pores, as measured by pressure drop over a given time interval. The lower the pressure drop, the narrower the pore and the more retentive the filter. The principle underlying this integrity test is the same as the basis for mercury intrusion into porous materials, except that pressures too low to cause polymeric pore distortions are involved. Non-integral filters will actually have water forced into them; integral filters will not. The automated test machine is sensitive enough to detect the small pressure decrease that accompanies the incipient entry of water into integral filters.

Since the water intrusion test involves wetting phenomena, it will reflect, in common with the other integrity tests, the influences of surface tension (therefore, water purity), temperature, and pore wettability, as already discussed. The importance of these factors in the operations of the bubble point, diffusive airflow, and pressure hold integrity tests has been detailed by Scheer et al. (1993). Filter manufacturers offering the water intrusion test also specify the maximum allowable water temperature and the water quality necessary to obtain an appropriate test result. Elevated water temperatures and contamination can lower the surface tension of the water, and in this case the test pressure required to push the water into the hydrophobic membrane will be lower. For this reason, as in all other integrity test methodologies, test specifications have to be defined and kept.

Redrying of Air Filters

The reuse of air filters is common because their utility is so little compromised by the particulate deposits they accumulate in any individual application. Before reuse, the air filter is sterilized, customarily by steaming. The water film that is deposited on the membrane surfaces by the condensation of steam is removed by drying before reuse. This must be done to discourage organism growth and penetration. In this regard, membrane surface hydrophobicity is a prime consideration; the water requires being removed. This is more easily done for PTFE filters than for those composed of any other commercially available polymer, including PVDF. A comparison of blow-downs used to restore full airflows to hydrophobic membranes after steaming showed that PTFE cartridges achieved same in 10–12 min. The PVDF filter was at only 25% of its initial flow rate by that time. The PTFE cartridge construction contributes to this result, but the greater avidity of PVDF for water retention is a significant consideration. The more extreme hydrophobicity of PTFE allows for its more rapid drying.

Advantages and Disadvantages of Water Intrusion Testing

The water intrusion test offers several advantages. For example,

1. The test is highly sensitive because its test pressures are in the range of the WPP of 0.45 μm rated filters.
2. Contaminants such as solvent mixtures are avoided.
3. In addition to the integrity, the validated hydrophobicity is tested, i.e., any contaminants on the membrane can be discovered.
4. The test can be performed in place, after steam sterilization.
5. Test times are greatly reduced, because contaminants do not have to be flushed off and in-place testing is not necessary.

The specifications defined by the filter manufacturers have to be observed to achieve reliable test results. In most cases the test is performed with automated test machines. This may be considered a disadvantage because of capital costs incurred. Nevertheless, automated test machines are usefully versatile and are also commonly used to perform other integrity tests such as the diffusive airflow and bubble point tests.

PREFILTER INTEGRITY TESTING

Use of Prefilters

The interruption of a filtration by an insufficiency of filter area (EFA) can be avoided by the use of prefilters. The purpose of the prefilter is to accept part of the particle load, which otherwise would deposit on the final filter. A particulate deposit over a given area of final filter could indeed be large enough to interfere with or actually block further flow. The sacrificial role of the prefilter in accepting its share of the particle load spares the final filter. If the combined prefilter-final filter assembly is sized appropriately, (utilizing flow decay measurements), the concentration of the accumulated particles per unit area would be reduced to where the flow would not become unduly restricted. Essentially, the prefilter would serve as an enlargement of the final filter area. The final filter's service life would be prolonged to where it would suffice for processing the entire preparation. In the pre-selection of the final filter, its ability to retain the entire load of the subject suspension without compromise of its final filter function would have been ascertained.

The purpose of the prefilter is to retain part of the total suspended solids load. While its efficacy may be more or less, its pore sizes and their distribution will govern the number and sizes of the particles retained by it and-or passed through it to the final filter under the selected operational conditions. The prefilter presumably serves no other purpose. If it were, for example, also being counted upon to remove some particular entity in its defined entirety, then it would, in that respect, be a final filter. Its "final filter" status would, in that case, require its own validation.

Validation requirements for prefiltration commonly restrict itself to the testing of the compatibility of the filter to the product and process conditions. Such testing is performed by the evaluation of leachables from the filter and-or particulate matter.

The number of these larger pores is unknown. Pores may be large enough for organisms to penetrate them, but may be so few in number as to yield airflows small enough to escape immediate notice. The perceived bubble point is almost certain, therefore, to be higher than the intrinsic. The difference is in the nature of a safety factor against bubble points that are too low.

Integrity Testing of Prefilters

The final filter as a consequence of its structural features and of the filtration conditions employed, imparts by its performance a distinct, and ultimate quality to the effluent. Its elimination of particles, including organisms, from a suspension, impacts the practical factors of rates of flow, and throughput volumes. The unique constructions of the final filter with regard to its porosity aspects, such as pore sizes, are depended upon to furnish the desired level of organism removals. It is these singular structural features that must remain unchanged over the entire filtration in order for the final filter's operational effects to be realized. It is for this reason that its pre- and after-use integrity testing is required.

Prefilters can effect organism removals, but their nominal ratings are relied upon only in a subsidiary fashion. It is the filtration conditions, chiefly the rates of flow, the products of the motivating differential pressure, that govern a prefilter's organism removal. These factors are assessed in the process validation step; they are not in the purview of integrity testing. Nevertheless, for sake of discussion, let us assume that the prefilter's structure can be subjected to integrity testing, and that its failure would result in a higher bacterial challenge to the final filter. Would that so affect the final filter as to compromise its organism retention? By definition, the final filter is qualified to be a sterilizing filter by way of the classic $1 \times 10^7 \text{CFU/cm}^2$ EFA *B. diminuta* challenge. On that basis, the final filter's organism retention capability would not be affected even by the full brunt of the organism load. Therefore, prefilters do not require integrity testing.

The prefilter function is the prolongation of the final filter's service life by sacrificially assuming part of the total suspended solids load. Integrity testing of prefilters is of interest because its compromise threatens the effluent quality with regard to organism content. Validation requirements for prefiltration are commonly restricted to investigating the compatibility of prefilters and fluid vehicles under process conditions, and to the evaluations of leachables. However, these considerations are evaluated in connection with choosing the membrane. They are independent of responsibilities addressed in the process validation. They would be dependent upon a filter's structural modifications only were they to permit organism penetrations of the filter along with compromise of the final filter's efficacy in organism retentions. Such might result if

changes occur to the pore sizes and/or their distributions. Such alterations may increase the number and sizes of the particles passed through to the final filter. The final filter's flow rate and throughput may be affected accordingly, but not the integrity. Therefore, the integrity of the prefilter requires no confirmation. If, however, the final quality of the effluent, depended, for example, upon the remove of some particular entity, such as endotoxin, then it would, in that respect, be a final filter. Its "final filter" status would, in that case, require its own validation including integrity testing. Also, the concern regarding extractables from the prefilter warrants being addressed. But these, too, being water soluble, are most unlikely to be affected on the basis of structural changes discernable by integrity testing.

On occasion there may be a need for the filtration activity to be completed within a given time period; whether because of production schedules or concerns of grow-through. FDA in 1976 addressed the problem of grow-through by requiring that the mixing and filtration of a preparation be completed within the eight hour period of a single shift, an interval too brief for grow-through to take place. Alteration of drug quality is not the concern. Processing time is the focus of this situation. Assuming a delay caused by failure of the prefilter to sufficiently spare the final filter its burden of particulate matter, the failed effort to meet the time limitation, or its success in meeting the goal is the defining determination. Either outcome could be indicated by a clock; integrity testing would be superfluous.

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12
Filter Manufacture Quality Assurance and Validation

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INTRODUCTION

The majority of sterilizing grade filters, as well as pre-filtration devices, are supplied into the highly regulated biopharmaceutical industry by outside vendors. This means that quality standards pertinent to the processes of the biopharmaceutical industry have to be applied to the vendors' processes. These standards start with the qualification of the production equipment during the development phase, and extend through the validation of the production process in its entirety, definitions of in-process controls and documentation during the production process, release criteria, specifications and tolerance settings, and complete traceability of the finalized product and product components. Standard operating procedures and training matrices are as manifold within the vendors systems as with the equipment end-user.

Besides validating/qualifying the entire process, vendors are also asked to deliver qualification documentation that supports the process validation requirements of the filter user. Depending on the equipment, such qualification documentation can be elaborate due to the sub-components of the device.

Once the equipment is ordered or supplied to the end-user, most commonly the vendor will submit qualification documentation as described above, provide support for qualification and acceptance testing, and in some instances offer product- or process-related validation services. The quality of the vendor's production processes often mirror the production processes of the relevant industry of the vendor's customer base. Additionally the vendors establish appropriate technical support structures to be able to react rapidly to any support needs of the industry. This is of importance as the end-user has to be able to answer regulatory enquiries or when equipment requires maintenance, calibration or repair. A production stand-still cannot be tolerated as it would resolve in multimillion dollar losses in revenue and put drug product batches at risk.

VENDORS DEVELOPMENT CYCLE

Vendors strive to improve their products and processes to be able to supply the industry with state-of-the-art equipment and improvements within the industries processes.

For this reason vendors constantly invest 3% to 8% of their revenue in the development of new product or in improvement projects of existing products. However, every time a product is newly developed a documentation trail has to be established, similar to that of pharmaceutical R&D.

Raw Material Supplies

The development cycle begins with the choice of a qualified sub-supplier and ends with a fully qualified product and validated production process. Vendor's development groups are multifunctional teams, which work together with sales and marketing, the supply chain, and production to have an appropriate scope of what is required within the supplied industry. For example, supply assurances of raw materials have to be given over a long time span; the required raw material must be readily obtainable at the quality specification; and the production capacities, as well as machineries, must be available. Once these cornerstones have been investigated and verified, the development of the product can start. Any effort to develop a piece of equipment without the knowledge of market need, supply assurance, and production feasibility is a wasted effort. These cornerstones are the first milestones which are documented within a development process and will require audits by the vendor's quality assurance and supply chain departments of the raw or sub-material supplier. These audits have to be well documented and are usually applicable to a minimum of two suppliers. Supply assurance for a vendor is just as important as for the end-user, as any supply change will result in a change notification and comparability studies, and, in some instances, a possible revalidation of the equipment at the end-user level and notification of the regulatory authorities. Therefore, changes must be avoided. Critical raw materials and components fall under long-term supply assurance contracts and might have multiple year inventory levels within the sub-supplier or vendor level. Additionally, vendor development will involve quality assurance to analyze whether the sub-suppliers quality certification, systems and assurance meet the specification given to vendor's development by the industry. As the end-user audits the vendor's processes, the vendor will do the same at the sub-supplier level. The more thorough the vendor's internal and external quality and supply investigations, the better the supply quality to the end-user.

Prototype Testing

Once the sub-supplies have been established, the vendor's development group will create prototypes and different versions of the equipment, which will be first tested in-house and at a later stage at a beta-site, which is commonly an end-users process development or small scale site. However, any stage of the pilot scale production and/or assembly has to be thoroughly documented to assure consistency and improvement, if necessary. In instances of the development of source code within equipment, any development of such code or any change within the code has to be documented in name of the programmer, date, description of change or progress (Fig. 1). The entire source code establishment needs to be auditable and well documented.

Prototypes will be tested and if these do not meet the specified requirements, the developers are required to go back to the drawing board and improve the equipment to users' specifications. It is highly important to test the equipment thoroughly, as it has to meet user requirement specification optimally, but also must have reliable, repeatable performance realization. The test data obtained will also help for validation purposes, for

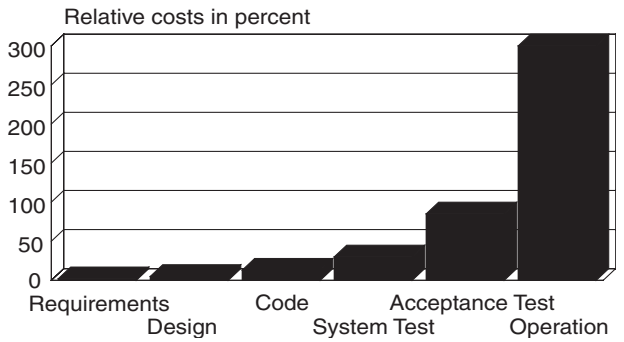


FIGURE 1 Costs of software development.

example when the system is running out of spec and what happens when it does so. As in any pharmaceutical process, the vendor processes are also defined within a specification band or parameters, in which the process has to stay within. However, during the development phase and small scale testing, the outer edges of the specification band will be checked to see what how fast this event could happen and what the result would be. Once the specification band has been determined it will be locked in place and all process parameters and settings will be defined within a standard operating parameter (SOP) structure.

Validation–Qualification

When finalized, the raw material quality, product parts production, assembly, and packaging specifications must be locked. This means that production parameters and tolerance are recognized and set, most commonly by repeated production batches as required within the industry itself. For example, machine setting, product formulations, production environments and flows have been defined and personnel trained accordingly. The locked-in parameters only create the assurance of reliable repeatability of the final product quality specification. The setting of production parameters also creates opportunities for analytical technologies to measure product specification during the production process. Process controls help to assure that the process is stable and performs as defined. When all specification, process requirements, and controls are defined, validation protocols and SOP have been instituted. Validation tests are commonly set by publicly available international standards, for example sterilizing grade filters have to meet current pharmacopeial requirements and will be tested accordingly. In the case of sterilizing grade filters, qualification tests are commonly performed, however these may vary from vendor to vendor:

- USP Plastic Class VI
- endotoxin
- particulates
- oxidizable substances
- pH/conductivity
- integrity test limits correlated to bacteria challenge tests
- steam sterilizability
- physical dimensions
- operating parameters, like max. operating pressure/temperature
- flow rates

Nevertheless, the vendors have their own sets of tests, which equipment will undergo to verify performance criteria set by the vendor and user alike. For example, in some instances pulsation resistance is also tested or specific extractable tests are performed. The extractable data as well as all other tested parameters are conducted with model solvents and specific lab test conditions. Often one could utilize the vendor's specific tests as indicators to check how the equipment might behave within certain environmental conditions. However, the indicator results obtained during the vendor's qualification stage have to be verified by appropriate process validation. The results of the qualification tests can be found within the vendors qualifications documentation (validation guides), which are supplied to the end-user (Fig. 2). In some instances, vendors also supply specific documentation which elaborate on, for example, extractables or asbestos fiber capture/release (very specific for Germany in the past). Filter vendors also supply quality certificates with every filter, which list release criteria and the attainment of such.

The qualification documents, however, will not replace process validation or performance qualification (PQ) at the end-user's side. These documented tests establish the basis requirements for the equipment to (*i*) be able to work within the biopharmaceutical environment and (*ii*) verify that the equipment meets regulatory requirements. If this scientific basis will not be met by the developed product, the product will be scrapped.

Quality Control Parameter

Qualification tests and the validation of the vendor's production process will also set the standards of tests which quality assurance will use to determine product consistency and reliability. Most commonly vendors have already standard quality assurance tests defined by other production processes or equipment specification. These can be utilized to a large degree; however, it could be that a specific piece of equipment requires additional tests or release criteria. For example, the in-process controls and release criteria for a sterilizing grade filter will differ from a membrane chromatography device or filter housing. Nevertheless, all the product categories must have appropriate controls and release criteria established to meets quality and consistency standards.

The vendor's development department must work in close conjunction with multiple departments. Development not only creates a new or improved product, but has to assure sub-supplies, determines appropriate production specifications, tolerances and the validation of such, which is commonly done with supply chain and quality assurance functions. Additionally, with the validation of the production process, close collaboration with quality assurance is required to create appropriate SOPs, validation and qualification documentation and the review of the development documents, especially for source code. Quality assurance works in close conjunction with development to assure consistent feasibility, performance, and quality. Finally, the vendor's product management and technical service departments are supplied with performance data and specifications of the new product by the development department. These data have to match or exceed the criteria set by product management, i.e., the end-user.

VENDOR PROCESS QUALIFICATIONS

Depending on the complexity of the vendor's products, the production processes require process-specific validation. Most commonly vendor production processes are multi-step,

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FIGURE 2 Filter qualification documentation example. *Source*: Courtesy of Sartorius Biotech GmbH.

meaning every step requires validation, appropriate operation procedures and qualification, and training and certification of the personnel involved.

Example Filter Manufacturing

For example, membrane casting for a sterilizing grade filter is one step within the production of a sterilizing grade filter (Fig. 3). This casting process requires very specific environmental process conditions and machine settings. These conditions can be humidity, temperature, solvent vapor saturation, casting belt speed, dope thickness, etc. During the casting process, the machine parameters are constantly monitored and samples of the casted product are taken and tested frequently. Even before the casting process, the dope mixing and ingredient concentration determines the success of casting a specific membrane property. The component recipes and mixing parameters are highly guarded, as these are the critical success criteria which will not only determine the feasibility of a cast, but also of the later configuration. However, the parameters and recipes have to be well defined and controlled to create reliable repeatability; the same holds true for the casting process.

All parameters and test results are documented within the batch records of the particular cast and can be reviewed by auditors. The documented results also serve as a historical database to perform statistical evaluations, evaluate process performance, or support development efforts. The casting process parameters determine the pore size of a membrane, but also its pore size distribution, i.e., the process has to be closely adjusted and monitored to achieve a narrow or desired distribution.

Once the membrane batch is cast, it will be pleated, sealed, end-capped, welded, integrity tested, bagged, and autoclaved again. However the procedure or individual production step line-up does not matter—every single step has defined process parameters in which the production step has to be run. The timeframe between every step requires as much monitoring as the step itself. The process has to be validated as an individual step and entirety.

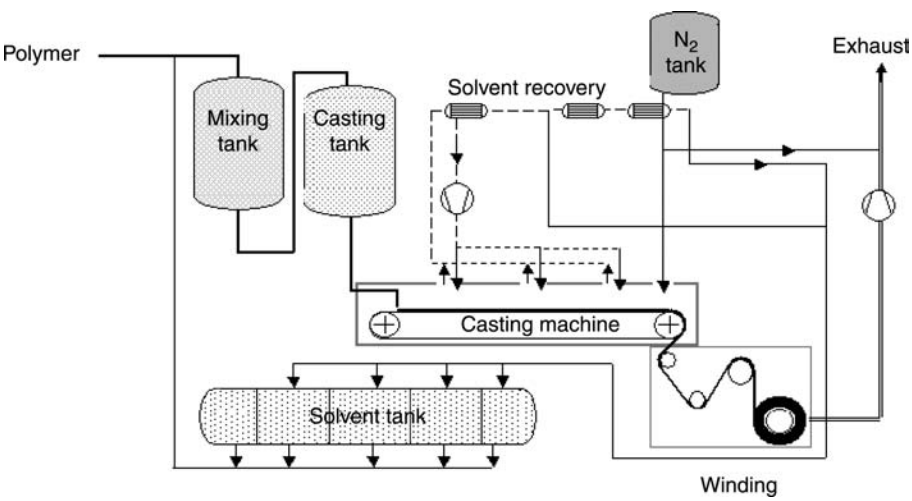


FIGURE 3 Casting process. *Source*: Courtesy of Sartorius Biotech GmbH.

This means that production parameters and release criteria are defined for each step and described within SOPs. Every parameter, specification, and tolerance level are documented within a validation master protocol and cannot be changed without approval by multitude of departments, most important of which is quality assurance. Release criteria are established by in-process tests. These tests can be destructive and non-destructive integrity tests, tensile strength, NVR (non-volatile residue), physical dimensions, pore size distribution, flow, throughput, etc. Once the criteria are met the product can move to the next step of the process. However, if the product does not meet the criteria, an investigation will be initiated to analyze why the product is out of specification. Most commonly such investigations happen at the development of full scale production, since the products are moved from development's pilot scale to full scale. Scaling within a vendor's process can be as difficult as in the end-user's industry. This might be seen as a negative, but should instead be seen positively as it is better to amend root causes of undesired product quality within the production validation process, instead of established production processes.

Once the production processes are established, maintenance protocols will assure that the production equipment is at standard to fulfill the criteria set. Maintenance protocols are written during the validation phase as wear and tear can vary and specific tools are constantly inspected during the validation phase.

Example Automated Processes

Any automated equipment utilized in the production process has to undergo installation, operation and PQ, especially regarding the process and systems controls. For example an injection molding system that is utilized to produce a specific part for a medical device requires as much qualification work and documentation as an autoclave within the pharmaceutical industry. The product component delivered by the molding machine has to be of consistent quality, complying with set tolerances and specifications. If any of these specified parameters is not met, the batch will not be released and an out-of-specification investigation will be performed. These tests are described and used as release parameters for validation batches and thereof commercially marketed batches. However, consistency in set quality parameters is the most important aspect in any stage of the production process.

Example Manual Processes

Certain production processes cannot be automated and the production step is performed by personnel, for example, welding. The settings within welding can be described, but only as indicator specification; for example, the tube volume, material thickness which will determine the protective gas pressure and welding energy settings. However, due to the complexity and individuality of some equipment, for example bioreactors or cross-flow systems, most welding might be done by the pure experience of the welder. The welders require specific certification and most often have many years experience. The welding itself will be analyzed and inspected before release, but this does not minimize the skill level required for such welding tasks. Similar skills are required for cutting, honing, bending, polishing etc. The validation within these processes is the certification and training of the personnel, log books, as well as the quality of the raw materials used. Any raw material entering the facility will be inspected and documented, and requires specific certification and log numbers. The raw material has to be traceable and of specified quality. Furthermore release quality tests and the database of these release

successes will also ensure that the personnel stays consistently within the quality specifications set. Release tests are of very high importance in any manual production process, as these are the most valuable qualification data points. A person cannot be validated; therefore all parameters surrounding the personnel have to be stringently monitored.

Example Packaging

An additional piece of validation work on the vendor's part is packaging validations and tests. The goods will be packed in specifically designed packaging which assures robustness during transport. The vendors will test the packaging design using specific standards, e.g., ASTM D 4169 and D 4728-95 (ASTM, 2003). These tests are drop and vibration tests. DIN ISO 12048 is a compression test, which will verify the stability of the packaging (DIN, 1994). As soon as the goods leave the factory, the vendor loses control over the handling of the goods. Therefore packaging plays a major role to maintain the quality and integrity of the goods shipped. Moreover, robustness is not only attached to mechanical stability, but also to thermal and chemical stability. Temperature changes during transport are not unusual, especially during overseas shipments. The packaging must be flexible enough to overcome any thermal expansion or shrinkage. It also should repel any condensation occurring due to temperature changes or changes in humidity. Oxidation due to sun light is probably the most common photochemical attack to polymeric packaging. The packing has to be stabile under these circumstances; otherwise polymeric degradation would result in weakening the packaging or particulate shedding of the packaging. This is especially important for gamma-sterilized packaging or products, as the packaging and product can degrade over time after that sterilization process. For this reason, shelf-life studies are routinely undertaken with gamma-sterilized goods. The shelf life will give guidance for the end-user and should be observed to avoid any increase of particulate or extractable matter.

The ultimate tests for packaging are multiple shipments into the different regions receiving the product via different carriers. At the end these tests will create a grid of test data of different means of transportation at different environmental conditions, which will result in a tolerance band for the designed packaging. Only by such tests can practical data verifying the experimental lab data be created, as the transportation process is often not in the control of the producer. The transportation process can often not be specified or defined by a user leaflet, i.e., the handling is unknown and the only option to verify that the transport is not doing any harm to the packaged good is the actual test.

VENDORS IN-PROCESS CONTROLS AND RELEASE CRITERIA

Depending on the vendor's products the in-process controls and release criteria vary from narrowly defined step-by-step controls within the production process to an end result control and release (Jornitz and Meltzer, 2001). Most commonly individually produced components are tested when produced and again when the individual components are assembled. As previously described the control and release criteria and tests are established within the development process and depend also on the criticality of the product supplied to the end-user. In some instances control and release criteria are fairly simple and encompass only a single test criteria; most of the time, though, product distributed to the pharmaceutical industry undergo multiple tests within the parts and final product production process.

Raw materials supplied to the vendor are checked first to determine whether the quality documentation is complete. Again, depending on the criticality of the component, the material might undergo specific tests to verify that the quality standards described are met. For example, polymer granulates undergo thermal profiles to check that the quality and type are the same as specified by the vendor to the sub-vendor. Granulates which are used for pharmaceutical purpose also undergo specific tests like endotoxin, particulate, and extractable tests. In other instances, the raw material is visually inspected; for example, stainless steel tubing in regard to surface finished, damages, flaws, and material stamps.

If the raw material does not meet just one of the specifications, the material will not be released into production. All raw material batch records are kept with the batch records of the resulting product. The product has to be completely traceable to allow appropriate investigation, if necessary. Raw material suppliers are generally audited once a year, depending on the significance of the raw material supplied. However, if there has been an incident the supplier will be audited immediately thereafter and corrective action verified. At that point the audit frequency could also increase until the reliability level required by the manufacturer is achieved.

For example filter cartridges, whether pre- or membrane filter, are tested for extractables (Fig. 4) to check whether there is any change within the profile, which might not meet release criteria (Jornitz and Meltzer, 2001; Meltzer and Jornitz, 1998). Similar tests are flow, throughput, mechanical and thermal robustness. Membrane filters are commonly individually integrity tested before release.

Stainless steel products also have specific definitions, which need to meet the biopharmaceutical requirements (Jornitz and Meltzer, 2001). These are individual stamping of the steel goods, welding certification, material qualification, and certification. The steel source can determine the quality of the steel. The steel

Identified Extractables Filter Cartridges from Several Filter Manufacturers

Cartridge A	Cartridge B	Cartridge C	Cartridge D	Cartridge E*	Cartridge F*	Cartridge G*	Cartridge H*
Dietyhlphthalate	Cyclohexan	Propionic acid	Dietyhlphthalate	Acrylic acid	Dimethylbenzen	Etherthioether	Caprolactame
Stearic acid	Ethoxybenzoic acid	Diphenylether	12 oligo. aliphates	2 phenolic oligo.	Etherthioether	Propionic acid	Butyrolactone
2,6-Di-tert.-butyl-cresol	2,6-Di-tert.-butyl-cresol	2,6-Di-tert.-butyl-cresol	Hydroxybenzoic acid	2,6-Di-tert.-butyl-cresol	2,6-Di-tert.-butyl-cresol	2,6-Di-tert.-butyl-cresol	Laurinlactame
2,2-Methylene-bis 4-ethyl-6-tert. Buty phenol	Cyclohexadiene 1,4-dion	4-Methyl-2,5-cyclohexediene-1-on	Tert.-butyl-methyl-2,5-cyclohexadiene 1-on	3 oligo. Benzyl-di-phenylmethan	3,5-Di-tert. butyl-4-hydroxyphenyl propionate	3,5-Di-tert.-butyl-methyl-2,5-cyclo-hexadiene-1-on	Laurinlactame derivate
Hydroxybenzoic acid	Phenylisocyana	Hydroxybenzoic acid	2,4-Bis(1,1-di-methoxy-1-ethyl)-phenol	Triphenylphosphite	2 N-containing high MW compounds	4,4-Dichloro-diphenylsulfone	4-Methoxy-4-chlor-diphenylsulfone
7 oligo. Siloxanes	Palmitic acid	Palmitic acid	Succinic acid	Stearic acid	Acetamide	Benzothiszolone	Adipinic acid
Bis-(2-ethylhexyl)-phthalate	Stearic acid	Dimethox/dipheny sulfone	3 oligo, siloxanes	Bis-(2-ethylhexyl)-phthalate	N-cont. aromatic high MW comp.	4-Hydroxypropyl-benzoat	Dibutylphthalate
12 oligo. Aliphates	12 oligo. aliphates	11 oligo. aliphates	Polyether	Polyacrylate	3 oligo. amides	6 oligo. Aliphates	Ethylhexylphthalate
4-Methyl-2,5-cyclohexadiene-1-on	11 oligo. siloxanes	Methoxy-4-chioro-diphenylsulfone		Ethylacrylate	Bis-(2-ethylhexyl)-phthalate	Hydroxyphenyl acetamide	Dihydroethyl-phthalate
	Methyl-4-hydroxybenzos	Bis-(2-ethylhexyl)-phthalate		Diphenylphthalate	10 oligo. siloxanes	Methoxy-4-chloro-diphenylsulfone	2,6-Di-tert.-butyl-cresole
	Etherthioether	Polyether		9 oligo. Siloxanes	2 oligo. aliphates	7 oligo. Siloxanes	Disobutylphthalate
		7 oligo. sioxanes		6 oligo. aliphates		Cyclohexanone	Diacetylbenzene
		2,4-Bis(1,1-di-methoxy-1-ethyl)-phenol					Cyclotridecanone
							4-4-Dichlorodi-phenyl-sulfone
							Propionic acid
							4 oligo. siloxanes
							3 oligo. aliphates

*Identification of the RP-HPLC peaks by FTIR is still in progress—extractables list of marked cartridges may be incomplete.

FIGURE 4 Table of extractables of eight different sterilizing grade filters.

components are required to be accurate, as these determine welding quality, corrosive robustness, and the electrolytic behavior within a system. Nowadays stainless standards are set by the industry, which define for example the ferrite content or surface roughness.

Depending on the application, the stainless steel equipment used differs greatly in the surface treatment. The smoother the surface, the greater the treatment steps and the costs involved. In some instances surface treatments are not needed or are even undesirable, and potentially a glass beaded surface is sufficient. However, since cleaning is a major factor within the biopharmaceutical industry, the surfaces are required to be smooth and with minimum groove rate. Any groove would allow pockets of microbial growth, which could potentially result in biofilm formation. Electro polishing, after high grid polishing, is utilized to cut any high peaks of material and avoid pockets (Figs. 5 and 6).

Electro polishing will remove any peaks or valleys within the surface structure. This is of importance as a buffing process after the electro polishing process could result in covered pockets which could result in microbial containment spaces. These pockets or caves could potentially shield microbial contaminants and would therefore be a starting point for biofilm formation growing out from that point. Therefore, any stainless steel pre-treatment, abrasion, buffing or polishing process has to be well qualified and must be adapted to the steel quality at hand and the end use of such equipment.

Most commonly, when automated equipment is supplied, appropriate qualification documentation is required before the equipment is released and shipped to the client. Without such documentation the equipment would be of no use and the shipment might be rejected. It is essential that these documents are send to the client for pre-approval. Once the approval is received only then can the vendor ship the equipment to the client. Appropriate qualification documentation is an essential release criterion nowadays. In instances of complex automated equipment, for example, fermentation equipment, factory acceptance tests (FAT) are crucial release criteria before the system is shipped to the client. These tests encompass a full-scale run of the equipment to see whether the equipment performs to user requirement specification. If there are points of concern,

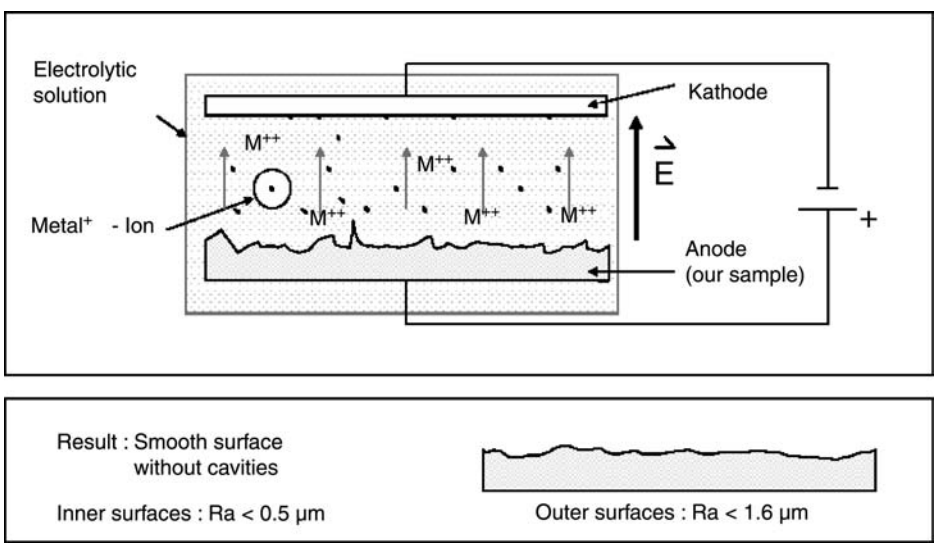


FIGURE 5 Schematic of an electro polishing process.

No.	Designation of procedural step	Remarks	Recommended abrasive	Grit	Peripheral speed in m/min
1a	Preliminary rough polish ("fettling")	Preliminary step for rough welds; only for very coarse work; recommended follow-up step: 1b, with 60-grain abrasive	Preferably grinding wheel with hard rubber or plastic bond	24 / 36	1,200 - 1,800
1b	Rough polish	First step for thick sheets, hot-rolled sheets or smooth welds	a) Grinding wheel with hard rubber or plastic bond b) Set-up wheel c) Grinding belt, if the shape of the piece permits	if 36 is necessary, follow up with 60	1,200 - 1,800
2	Finish grind	Standard step for cold-rolled sheet or coil	a) Set-up or rubber wheel b) Grinding belt, if the shape of the piece permits	80 / 100	1,500 - 2,400
3a	Precision grind	The surface finish corresponds to that of roll material in accordancewith "Procedure o (IV)"	a) Set-up wheel b) Grinding belt, if the shape of the piece permits	120 / 150	1,500 - 2,400
3b	Precision grind	Preparatory step in producing a normal polish following step 3a.	a) Set-up wheel b) Grinding belt, if the shape of the piece permits	180	1,500 - 2,400
3c	Precision grind	Intermediate step in producing a normal polish following step 3b	a) Polishing wheel b) Grinding belt, if the shape of the piece permits	240 abrasive paste for set-up wheel, or 240 grinding belt	2,400 - 3,000 Grinding belt: approx. 1,500
4	Brushing	To produce a smooth, matte, silk luster. This step, following one of the "o(IV)" procedures, produces a surface finish that corresponds to the designation "burnished." Brushing finer (e.g., high-gloss polished) surfaces produces a very attractive effect. The surface finish will depend on the brush speed and the abrasive used	Tampico	Abrasive paste made of pounce or quartz powder. Other abrasives may also be used, depending on the desired surface finish	600 - 1,500
5	Polishing or lapping	Final step for producing a normal polish follwing step 3c (Note: lapping leaves fine chatter marks)	Polishing wheel	Burnishing compound for stainless steels in stick or cake form	
6a	Polishing	a) Preparatory step for producing a high-gloss polished surface following step 3c	Polishing wheel	320 - 400 finish polishing compound in stick or cake form	2,400 - 3,000
		b) Preparatory step for producing a high-gloss polished coil	Polishing belt	Burnishing compound for stainless steels in stick or cake form	approx. 1,500
7	Blasting	Final step for producing a matte, non-directional surface structure	Glass beads Stainless steel grit Nonferrous quartz sand	various	

FIGURE 6 Table of different polishing methods and the end result.

these will be adjusted to fulfill the defined criteria. A release criteria, external to the manufacturer, but as critical is a site acceptance test (SAT) of this equipment. At that point the equipment is check at the client's site, under the environmental conditions of the client.

From an end-user standpoint, the release criteria of the vendor have to meet the risk assessment criteria set by the end-user (and more often the regulatory authorities). Depending on the quality impact of a specific component or equipment supplied, the release criteria on both sides, vendor and end-user, will differ in stringency. The quality of supplied water for injection (if not produced within the facility) has a higher risk attached to it than a condensate valve on a tank. Different risk or impact classifications have to be defined for product and equipment supplies (Fig. 7). Some products have a direct impact on the quality of the end product, some have only a minor influence, and some have no influence but are used to check on a component with a quality influence. For example, an integrity test system does not have a direct influence, but is used to check the integrity, i.e., quality of a sterilizing grade filter, which has an influence on the quality. Therefore the calibration of such test system is essential. The release and test criteria for these products will differ and be defined in a way which will meet the necessary quality purpose. It would make no sense to use similar evaluation conditions for non-critical items. It would just raise costs and possible process delays. Therefore these risk assessments have to be performed before release criteria are defined.

QUALIFICATION OF EQUIPMENT

Probably the most descriptive and most utilized guidance on qualification mechanisms is the GAMP (Good Automated Manufacturing Practice) guidance published by the ISPE

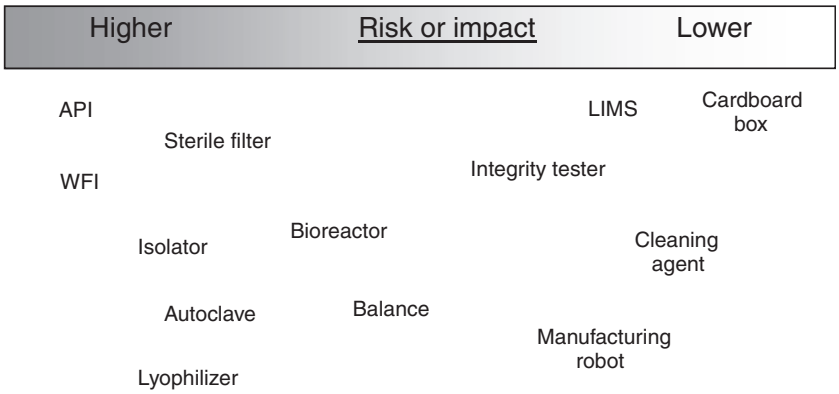


FIGURE 7 Possible example of risk and impact assessments.

(International Society of Pharmaceutical Engineering) (GAMP, 1998). It describes thoroughly the individual, necessary steps required to fulfill the quality expectations of automated systems. This guidance is used for a multitude of equipment utilized within the biopharmaceutical industry; for example, autoclaves, lyophilizers, filling machines, integrity test systems, bioreactors and others.

Within the GAMP documentation, specification steps are described but also three main qualification requirements—installation qualification (IQ), operational qualification (OQ) and performance qualification (PQ). There are other qualification tests which are quoted randomly; for example, design qualification (DQ) and system qualification (SQ). However the three major qualification segments are IQ, OQ, and PQ and these are applicable to every automated piece of equipment supplied.

A system design and the qualification steps all start with the user requirement specifications (URS). This is the foundation of any system to be designed and, if defined inappropriately, the entire project might be prone to fail or at least will require rework with additional costs involved. The URS can be seen as the foundation of a building: the better the foundation the better the construction on it. Any of the above-mentioned qualification steps are the verification of the URS, functional specification (FS) and design specification (DS).

Installation qualification: Documented verification that all important aspects of hardware and software installation adhere to the system specification.

Within this qualification, the entire system is checked to ensure that all components are correctly installed and that the entire documentation for the individual components is available. Most often the IQ step runs through a thorough checklist to evaluate that everything meets the requirements set within the design or hardware specifications (Fig. 8) (Jornitz and Meltzer, 2001; Meltzer and Jornitz, 1998; Spanier, 2001).

The IQ documentation is supplied by the vendor but checked by the end-user. A frequent and most practical practice would be to perform the IQ and OQ part during the FAT, which verifies that the system is working.

Operational qualification: Documented verification that the system operates in accordance with the system specification throughout all anticipated operating ranges (Fig. 9).

These tests verify that the FSs are met by empirically checking and testing, against the manufacturer's recommended test sequences, all the critical operational and functional features and performance specifications of the machinery. These test

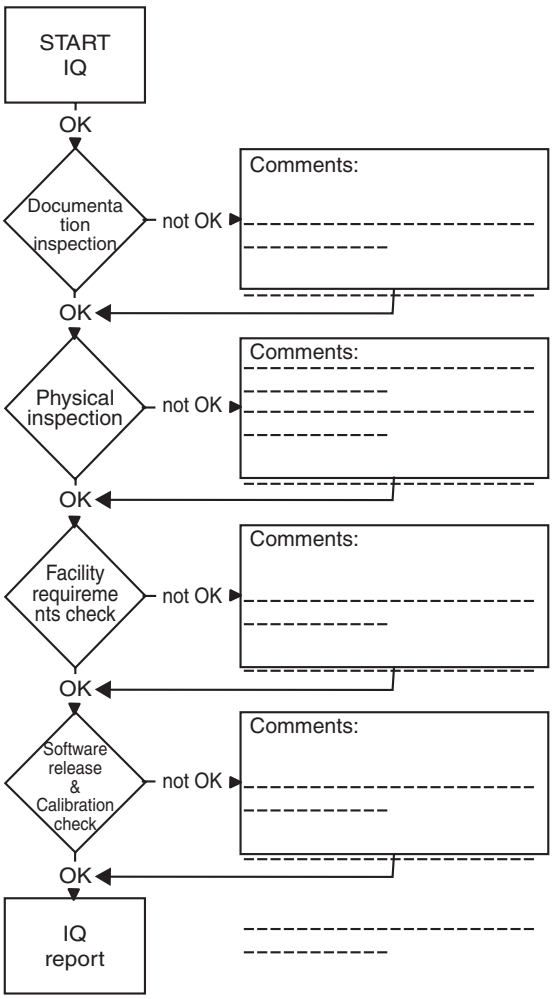


FIGURE 8 Typical flow diagram of the first layer of an IQ protocol.

sequences are performed within the vendor's facility, again most commonly during the FAT. Within this qualification phase the system will run at the specifications given by the user. Therefore, vendors are required to have all supplies necessary to run the system; for example, water and steam supplies. The OQ can be performed within few hours or weeks, depending on the complexity of the system. Most commonly the OQ documentation is already established within the process of the FS, as every single function described must be tested during OQ. If a FAT happened and the documentation was not established at this point, the workload will be tremendous and the precision will suffer.

Once the system is run through the FAT and OQ and all documentation is established, the system can be shipped to the end-user. At this point the PQ is performed as the final part. The PQ is often also part of the SAT or visa versa, depending on individual user procedures.

Performance qualification: Documented verification that the system operates in accordance with the User Requirement Specification while operating in its normal environment and performing the function required by the process to be validated.

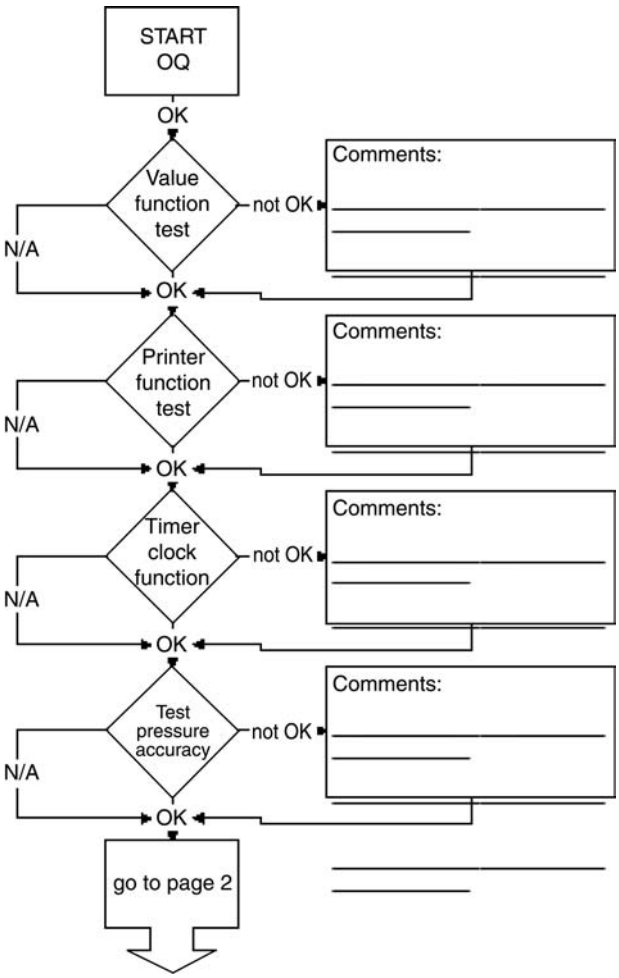


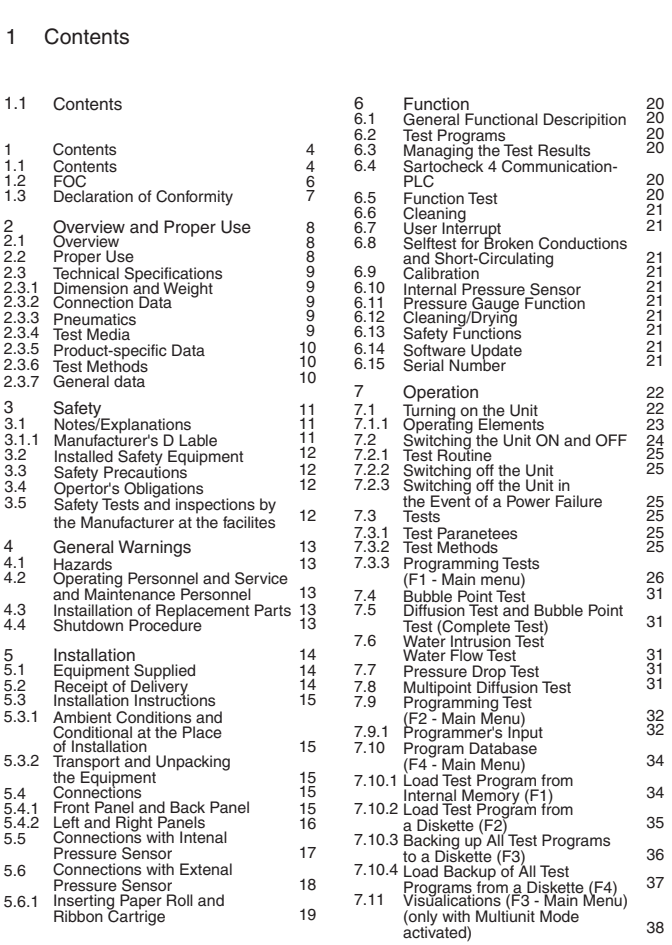
FIGURE 9 Typical flow diagram of the first layer of an OQ protocol.

These records include batch records, routine calibration, and performance checks, which are commonly defined by the equipment used. Every piece of equipment has different requirements of compliance with specifications defined within the specification phase. Moreover, the environment within the end-user facilities varies. For this reason PQs are used to check whether the equipment works within such an environment. Additionally, during the PQ phase, the equipment may be pushed to the limit to verify that it still performs and does not spiral out of control. In some instances automated equipment might malfunction when, for example, the software is pushed to a limit. It could well be that the system shuts down or that certain controls and adjustments elevate themselves out of control or set tolerances. These stringent tests belong to a risk assessment program, which determines the functionality of the system. Will it still work in as robust way as requested? Or will it perform in a way detrimental to the entire manufacturing process? The environment certainly has an influence on such functionality, as well as the process control system and its source code. It is sometimes the case that systems are not validatable because of commercially available software, adjusted to the purpose, but not fully compatible. Such software might not be able to cope with the stringency and demands of a production process and therefore would show insufficient performance.

These three fundamental qualification processes are repeated during each phase of the validation process. In the qualification phase a baseline level of performance information is obtained from the component manufacturer's data and test results, structural testing of the software, and the associated vendor documentation (Fig. 10).

Equipment validation packages must be prepared and available for the user's own validation efforts and tests to verify proper functioning of the equipment. These validation packages are commonly very comprehensive and cover every function of the equipment. For example, the documentation for a complex fermentation system can result in filling close to 1000 ring binders. In instances regulations applicable to the particular equipment will be quoted for the user to support other necessary validation or qualification processes within the facility. As described the equipment supplier can support, and commonly does, the end-user with installation and operational qualification documentation. However any process validation or PQ has to be performed within the facility and process environment. This will assure that the equipment is functioning properly inside the laboratory setting within the manufacturer's facilities.

Finally, maintenance and continued testing and verification are the responsibility of the end user, who may seek assistance from the equipment manufacturer or its own maintenance department. Establishmnet of a service manual is required before equipment



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FIGURE 10 Example of a validation documentation for an automated system. *Source*: Courtesy of Sartorius Biotech GmbH.

is supplied to assure appropriate maintenance possibilities. Such service manuals list spares required within specific frequencies. Commonly the vendor can predict at which interval certain parts of the system need to be exchanged or replaced. These essential spares need to be defined and listed within the service manual, as well as maintenance intervals. These tasks can also be performed by outside service organizations; however, the qualification of these organizations has to be verified. Most often service contracts are established between the vendor service side and the user maintenance department.

Another important aspect should not be forgotten—training. All qualification and acceptance steps are good, but will be useless if the staff utilizing the equipment is not trained effectively. Training protocols and SOPs need to be described before the equipment is used. Both training manuals and SOPs should be reviewed to assure correctness.

Stages of Equipment Supplies and Qualifications

Stages of the individual specification and qualification segments are mainly visualized within the V-model of the GAMP guidance (GAMP, 1998). The V-model shows the different responsibilities, but also interactions of specifications versus qualifications (Fig. 11). It is often modified to meet different requirements of different equipment suppliers.

Within the V-model, the individual tasks or steps are described, but also responsibilities defined. In some parts of the process the user is solely responsible; in other parts, the supplier is responsible; and, specifically in the qualification phase, the

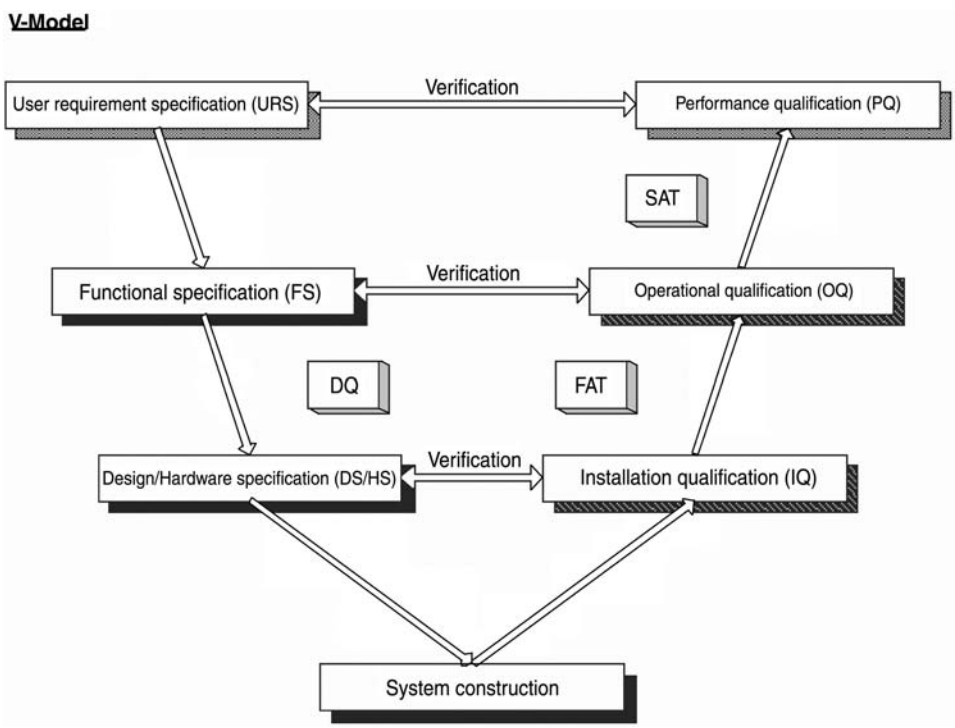


FIGURE 11 V-Model.

user and supplier share responsibilities, as most often these tasks are performed jointly. Every single step is of utmost importance and has to be viewed with stringency and thoroughness, as every subsequent step depends on the quality of the previous task. The entire system can only be as good as the starting quality, therefore multiple other process control and approval steps are involved, which are not shown within the V-model. However, before a system is built, each function, software and hardware design has to undergo critical review to verify that the user requirements and specifications are met. In some instances the specifications given might not be feasible to design or produce, or sub-parts are not available or too costly. Sometimes, a cost focus might be not desirable, as cutting corners might result in a system which is not fulfilling the needs of the process defined. Examples have shown that shortcuts in respect to equipment or design qualities have resulted in higher adjustment costs at a later stage. Inadequate attention to the design of the system has resulted in yield losses or dysfunctions. The costs resulting from such failures are tremendous. The recommendation has to be that the user and vendor work closely together to find an optimal solution for the particular need. Costs have to reasonable, but should not be the main focus.

The most important aspect is the user requirement specification, in which attention to detail is essential. A rough idea given as a URS will end up as a back and forth between the user and vendor in the FS stage. Valuable man hours are wasted, which is undesirable for both parties. Often forgotten but always present is that the user is the specialist of the application and the vendor the specialist of the equipment. Utilizing both sets of experience will result in the best possible option. However, controls and measurements should be utilized during the milestones to assure that the system will function once built and implemented within the facility.

As described, the V-model creates an overview; however project flows and detailed activity description require other tools, for example specific project management software (Fig. 12). These tools will define activities in detail and also cite control points for parts of a system, the entire system, or just the raw materials (Spanier, 2001; Technical Reprot, 1992; Wolber et al., 1988). The timeframes will also be reviewed on a frequent basis, as time pressures commonly will result in human error. Every vendor has experience with their equipment supplies, and knows what quality system requirements need to be established within a detailed project plan. These control points also help the vendor avoid any errors that would create additional costs.

CONCLUSION

Validation and qualification of equipment within the end-user facility under actual process conditions is an essential need and regulatory requirement. However, vendors of equipment, whether consumables or capital equipment, perform a multitude of qualification programs within their own facility. Such qualifications programs start during the development phase and commonly include not only the vendors' own processes, but also sub-vendor sites, processes, and product qualities. A vendor cannot just rely on the sub-supplies, but has to assure their quality just as any end-user needs to do. Furthermore, the development team receives quality milestones by the end-user. These specifications have to be kept, which means that within the development phase, control mechanisms are defined and are used to verify that the specifications are met and as a release criteria at full scale production. Similarly, capital equipment receives user requirement specifications which are converted into FSs followed by software/hardware

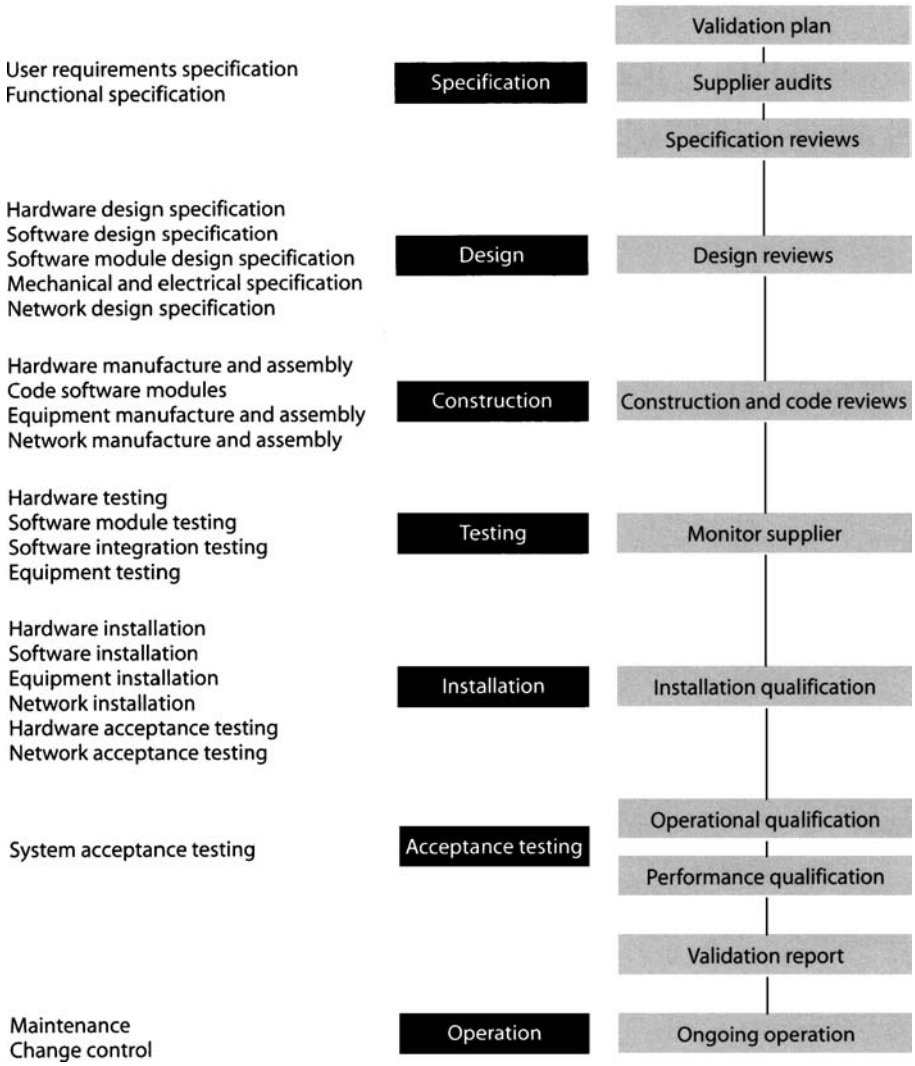


FIGURE 12 Validation schematic. *Source*: Courtesy of J. Spanier.

DSs. Again the fulfillment of the user requirement specifications has to be controlled at every stage to avoid any surprises and non-compliance. The capital equipment runs through different specification stages like a consumable product runs through a development phase. At the end of the day, both product groups require compliance to the user specifications.

Once the goods have been developed or built, the performance has to be qualified within the user's environment. Does the equipment perform under these circumstances? For example, sterilizing grade filters undergo process validation utilizing the actual or very close to the actual drug product and process conditions. Evidence has to be given and documented to show that the filter is performing to the set requirements under the environmental circumstances. The Performance Qualification stage accomplishes this for capital or automated equipment. Again the equipment might be pushed to its limit to

assure that it functions under worst-case conditions reliably. The tests are performed on-site to guarantee that any environmental condition does not have an adverse effect on the performance of the equipment. Lab tests at the vendor or pure certification cannot be accepted and will not meet regulatory requirements.

Vendors nowadays do not just produce and supply goods, but make sure that these goods meet the requirements of the biopharmaceutical industry and its regulatory authorities. Moreover, once an item is sold, the vendors' efforts do not stop; they support the end-user with services to support any subsequent user qualification and validation effort. Both the vendor's experience and end-users know-how will optimize the process reliability and, in combination, ensure that the specification of the equipment will meet the needs of the process. The vendor has to be more than halfway toward the end-user by supplying qualification data of the goods supplied, which can be utilized to either make a choice of equipment or be part of the filing documentation.

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Validation of the Filter and of the Filtration Process

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INTRODUCTION

The validation of sterilizing filtrations, and of the sterilizing filters involved is critical to the production of a sterile drug product, or of a sterile active pharmaceutical ingredient (API). The sterility of the drug preparation cannot be ascertained by analysis of its samples. It is impossible to test every drug container to assess its sterility. Similarly, a statistical determination of sterility would require so large a sampling as to be impractical. Validation of a process provides the assurance that its product is sterile. Such validation is a regulatory requirement (FDA, 1985). Validation of the filters used to achieve a sterile API is also necessary. It serves to assure that they perform in the manner intended; this may also be a regulatory requirement if there are no further sterilizing steps for the ingredient after its being formulated into the final product. Validation of a sterilizing filtration process used for pharmaceutical liquids essentially involves three things: determining the effect of the liquid on the filter, determining he effect of the filter on the liquid, and demonstrating that the filter removes all microorganisms from the liquid under actual processing conditions, resulting in a sterile filtrate (Madsen, 2006).

One further point may need to be considered, namely, the interaction among the contaminating organisms, the solution, and the filter. Therefore, several studies are necessary to perform a complete filter validation. Such would include investigating extractables, chemical compatibility, initial filter performance, and bacterial retention testing. If the filter is to be post-use integrity tested in product-wet condition then validation of product-wet integrity test specifications is also necessary. Reuse testing is indicated if the intention is to use the filters more than once. This latter practice is not encouraged because of the heightened possibilities of cross-contamination.

PRESENT STATUS

Until rather recently it was believed that the sterilization of fluids could unerringly be achieved by their filtration through a “sterilizing” membrane whose proper and pertinent identity was confirmed by its pore size rating, which was itself determined by integrity testing. Developments in filtration practices showed this belief to be too generally

founded. What had once seemed simple is now recognized as being quite complex. It was discovered that the positive conclusions based on pore size ratings were subject to modification by the physicochemical specificity of the organism-suspending fluid; by the individuality of the organism type in its size-changing response to the fluid; in the possible changes in pore size induced by the fluid's effect on the filter; and by the adsorptive qualities of the filter resulting from its particular polymeric composition; all influenced by the filtration conditions in their numerous varieties, but especially by the transmembrane pressure. A filter may not sterilize the same preparation under different filtration conditions, especially under dissimilar differential pressures (Leahy and Sullivan, 1978). A given membrane may or may not retain a particular organism type suspended in a different drug vehicle (Bowman et al., 1967). The organism type need not remain constant in size, but may alter in response to its suspending fluid (Gould et al., 1993; Leo et al., 1997; Meltzer et al., 1998). The effect of the vehicle upon the polymeric membrane may cause a change in its pore sizes (Lukaszewicz and Meltzer, 1980).

The certainty of obtaining sterile effluent requires far more than the identification of a "sterilizing filter" by a pore size rating. The complex of influences governing the outcome of an intended sterilizing filtration necessitates a careful validation of the process, including that of the filter (PDA Technical Report No. 26). The very drug preparation of interest, the exact membrane type, the precise filtration conditions, and the specific organism type(s) of concern should be employed in the necessary validation.

Given the complexity of the organism removal operation, it is doubtful whether a universal sterilizing filter can be devised. Certainly, there is no known absolute filter, one that will retain all organisms under all conditions, especially if viruses are included (Aranha, 2004). Therefore, the successful attainment of a sterile filtration with regard to specified organisms of interest must in every individual case be attested to by the documented experimental evidence that constitutes validation.

MECHANISMS OF RETENTION

The retention of organisms by filters is central to achieving sterile effluents. The mechanisms whereby particles are removed from solutions by attachments to filters are variously characterized. How the organism and filter surfaces encounter one another, as by the influence of inertial impaction, Brownian motion, or gravitational settling, etc are, in this writing, not as significant as the bonding that keeps the two in juxtaposition after the contact is established. The nature and strength of the mutual bonding that joins the two surfaces are the considerations of interest.

For the purposes of this discussion, each of two particular situations constitutes a mechanism. Sieve retention, also called size exclusion, size discrimination, or direct interception is the mechanism that is most relied upon, and that is operative in most filtrations. It results in particle captures because the particle is too large to pass through the restricted areas of the filter's pores. Mechanical in its function, it is, once joined, augmented by electrical effects derived largely from partial charges.

Adsorptive sequestration is the mechanism that retains particles small enough to enter and pass through the filter pores. Such small entities in their passage bond to the pore walls; thus effecting their retention. The adsorption of one solid surface, the particle, to that of another, the filter, is the consequence of electrically charged atoms. As is commonly known, like charges repel; unlike charges attract. Such unions can eventuate from full ionic charges such as are involved when quaternarized charge-modified filters

are used. However, these adsorptions usually result from the mutual attraction of partial-charges of opposite signs. Such bonding phenomena as permanent dipoles, induced dipoles, hydrogen bonding, van der Waals forces, and hydrophobic adsorptions may be involved (Meltzer and Jornitz, 2006; Gabler, 1978).

Given a liquid with a sufficient degree of loading, the quantity of particles retained on a filter's surface may build a filter cake. Depending upon the packing density of the cake it may, in effect, function as a filter overlying the microporous membrane. Either or both of the above described mechanisms may operate to remove particles from the liquid as it permeates the interstices of the filter cake. Some consider "filter cake" formation to be a particle retention mechanism. Be that as it may, its formation is not likely from the lightly loaded liquids being readied for sterile filtrations.

The FDA's 2005 Aseptic Processing Guidance (September 2004) states: "A sterilizing grade filter should be validated to reproducibly remove viable microorganisms from the process stream producing a sterile effluent." This emphasizes the importance of bacterial retention validation of the sterilizing filter. Bacterial retention validation provides data verifying that the filtered product is safe from a microbiological perspective. Bacterial challenge testing is performed by the filter manufacturer to classify the retention capability of the filter (PDA Technical Report 26). The filter user is expected to perform testing to demonstrate that the intended filter construction will completely remove a challenge organism from a product or product family under process conditions. The validation of the intended process filter should simulate "worst case" production conditions, including the size of influent bioburden, and filter integrity test values. "Worst case" conditions means those circumstances that are least conducive to organism removals.

Similarly, the effect(s) of the product on resident bioburden and/or the standard challenge organism may not be well recognized. Organisms grown under nutritional stress conditions may have different characteristics from those cultured in rich, defined media. Poor nutritional conditions, such as those in many drug products, result in smaller organisms than those grown under optimal nutrient conditions (Geesey, 1987; Gould et al., 1993; Sundaram et al., 1999; Meltzer and Jornitz, 2006). These observations should be accounted for during sterilizing filter validation testing.

Influences on Mechanisms of Retention

The retention of an organism by a filter represents a nexus of many interdependent factors. These include the filter pores, their numbers and size distribution, and restrictive diameters; as also the types and sizes of the organisms, their numbers, sizes, and structural make-up. It is the matching of the sizes and shapes of the pores and organisms that is the determinant in the sieve retention or size exclusion mechanism. It is axiomatic that a particle larger than a pore cannot penetrate it unless compressively deformed by excessive differential pressures. Although membranes are classified in terms of single pore size ratings, they are actually characterized by pore size distributions, albeit usually of an unknown magnitudes. Thus, it is conceivable that an organism that could be retained by a pore of one of the sizes characterizing the distribution would escape capture were it to confront one of the membrane's larger pores. Absolute retention is possible only when the smallest particle of the particle size distribution is larger than the largest pore of the pore size distribution. Given the usual application of filters, of unknown pore size distributions, to the removal of organisms of unknown size distributions, it would be inappropriate, and misleading to declare that a particular filter will be absolute in its action.

Sieving, the chief mechanism of particle removal, can be reinforced by the adsorptive sequestration of the particles. The electrical charges on the surfaces of the organisms and filters manifest mutually attractive forces. This interaction results when they are opposite in sign. The one surface will become adsorptively bonded to the other. The result is the arrest and removal of the organisms as the fluid flows through the filter pores. The polymeric composition of the filter matrix in terms of its polarity determines its surface charge and, hence, its tendency to undergo adsorptive effects. However, the physicochemical nature of the suspending liquid can so modify the electrical attractive and repulsive forces by its ionic strengths, pH, surfactant content, etc. as to promote or hinder adsorptions. Thus, the same organism and filter may interact differently in solutions of various compositions; a desired organism removal may or may not result. An example of such an occurrence was noted by Bowman et al. (1967): Using a 0.45-μm-rated mixed cellulose ester membrane, a given preparation could be sterilized by the removal of its *Brevundimonas diminuta* content. However, the addition of penicillinase to the same preparation prevents the attainment of sterile effluent. The proteinaceous component preempts the adsorptive sites of the filter, preventing the sequestration of the organisms. That adsorptive influences were at work was shown by the eventuation of a sterile effluent from the penicillinase preparation when a 0.22-μm-rated membrane was employed. Sieve retention is the effective mechanism when the tighter membrane is used.

The differential pressure is especially influential in its effect on adsorptive interactions. The higher it is, the faster the liquid flow and the shorter the residence time of the particle within the pore passageway. This reduces the particle's opportunity to encounter the pore wall and, thus, minimizes the likelihood of adsorptive sequestrations. Intriguingly, there is reason to believe that the concentration of organisms, aside from their total numbers, influence the attainment of sterile effluent (Zahka and Grant, 1996; Mouwen and Meltzer, 1991).

The filtration conditions also have an influence on organism removals. For example, the solution's viscosity may be high enough to frustrate an organism ensconced within the liquid stream from reaching an adsorptive site on the pore wall before it is carried out of the filter by the convective flow defined by a given ΔP. Given the reciprocal relationship of viscosity and temperature, the opposite effect can result when the same filtration is performed with the liquid preparation at a higher temperature.

Also, the ratio of organisms to pores can strongly affect the rates of flow and their consequences in terms of throughputs and filter efficiency. Thus, even the effective filtration area (EFA) may impact on the likelihood of obtaining sterile effluent.

The sterilization of a fluid by a filter depends upon a number of factors; some more important than others. Most influential are the relative sizes and numbers of the organisms and of the restrictive pores. The physicochemical nature of the fluid in terms of its ionic strength, pH, osmolarity, viscosity, etc. is another contributing factor. The possibilities for the adsorptive sequestration of organisms by the filter depends upon the polarity of both their surfaces as expressed by the partial-charge induced van der Waals forces. The polymeric nature of the filter may also governs its tendency to arrest certain organisms via hydrophobic adsorptions. The type, number, concentration(s) and especially the size of the organisms whose removal is sought relative to the numbers and sizes of the restricted diameters of the filter pores is of obvious importance in determining the extents of organism removals. The susceptibility of the filter's pore sizes to alterations by contact with given solutions bears consideration. The organism size is of obvious influence. It too may be altered, whether increased or diminished, by contact with fluids of certain compositions. The ingredients of liquid preparations such as

surfactants, various proteins, and charged entities such as colloids can affect the filter's sterilizing actions. The filtration conditions, eg: temperature, viscosity, and especially differential pressure will influence the outcome of the filtration.

The mechanisms of organism removal being chiefly sieve retention, the size and numbers of the organisms and filter pores are important factors: as also the pore size distribution. Incompatibility between fluid and filter may result in pore size alteration; while incompatibility of fluid and organisms may be the cause of morphological changes in the latter. In either case, the organism removal by size exclusion or sieving may be compromised. A proper combination of all these factors is required to produce a sterilizing filtration.

COMPATIBILITY OF FILTER AND LIQUID

The compatibility of filter and liquid should not be assumed. Testing for compatibility investigates whether the drug product has a deleterious effect upon the filter membrane or its support materials. The goal of compatibility testing is to investigate whether the filter maintains its pore integrity following exposure to the product under the process conditions, and to ascertain that the filter medium is not adversely affected by the product or process conditions.

The liquid vehicle can alter the properties of the filter, including its pore sizes (Lukaszewicz and Meltzer, 1980). The changes may derive from the relaxation of the casting strains undergone by the filter during its manufacture. The direction of the pore size change is unpredictable. Another consequence of plasticization could be a dimensional swelling of the polymeric matrix at the expense of the pore areas. Such occurrences could interfere with the expected pattern of sieve retentions. It is, therefore, required that the validation exercise assure that this means of particle removal, if operative in the filtration, remain reliably so during the entire filtration. A study of the filter performance is necessary to make certain that it remains unchanged and dependable during the entire filtration.

Gross incompatibilities, such as membrane degradations, for example, dissolutions, hydrolyses, oxidations, may easily be recognized from changes in the filter's appearance. However, subtle alterations in the filter's structure will require experimental investigation. This can be achieved by way of integrity testing to see whether pertinent porosity alterations result from the exposure of the filter to the drug solution. The rate of such structural change being unknown, the testing should be performed after exposing the filter to the liquid for a period of time at least equal to the duration of the filtration processing step.

Comparing pre- and post-filtration values of bubble point determinations would reveal whether pore size alterations in the larger pores, signaling incompatibility, was caused by the filter/liquid contact. Such may be caused by the plasticizing action of liquid molecules intruding among the molecules of the solid polymeric filter. An enlargement of the polymer's intersegmental spaces by the liquid's plasticizing action could alter the particle retention expected from the sieving mechanism. Diffusive airflow testing would, however, more generally disclose changes caused to any of the pores regardless of their size. The polymer's intersegmental spaces are related to the filter's pores. The liquid molecules' intrusions into these spaces could serve to convert them into larger pores. This conceivable occurrence necessitates experimental investigation to make certain that it is not a realistic possibility.

Extractables

Contact between the molecules of the liquid and those of the filter polymer may result in a leaching of molecular substances from the filter into the drug solution. Such substances may consist of remnants of the casting formulae used to produce the filters, and of additives designed to protect the polymer molecules against chemical degradations during their fabrication and application as filters. Included are stabilizers against the oxidation of polymer molecules such as polypropylene; against the dehydro-dechlorinations of polyvinyl chloride as caused by ultraviolet light, and oxygen; and to counter the physical degradations caused by the heating and shearing inherent in the milling, extrusion and molding involved in working with polymers. The possibility of extractables being added to the filter's effluent deserves experimental investigation. The presence of leachables or extractables are a concern. They may be harmful to the drug's recipient. Their identity and avoidance is sought if at all possible. The likelihood of releasing extractables into the drug preparation during filtration is increased by plasticization of the filter polymer. The filter's reduction in viscosity by plasticization speeds the migration of substances from within the filter into the drug preparation. The time interval over which extractables are to be assessed should match the exposure of the filter to the liquid. Securing enough material for identification of the extractables, usually by way of ultraviolet or infrared spectral analyses, can be accelerated and accomplished by refluxing the extracting solvent through an appropriate area of the filter by the use of soxhlet extractors.

It is necessary to generate extractable data to demonstrate that the filter does not add an unacceptable level of particles and/or chemical extractables to the product stream. Depending on the use of the filter, a variety of carriers may be tested. Usually the actual drug preparation is not used for extractable testing because of the possible interference of the product constituents with components extracted from the filter.

Water extractable data for filters are typically available from filter vendors. Typically, water extractable data are generated following a 4-h soak time of the filter in water. Additionally, filter vendors may generate water extractable data for filters after one or more sterilization cycles. Post-sterilization filter extactable data demonstrate the effect that sterilizations, either moist heat or irradiation, can have on the extractable level that are found. Since most (but certainly not all) pharmaceutical products are aqueous based, these extractable data have applicability to most products.

Further extractables validation may be required for filters used to filter API's that are used with non-aqueous solvent systems. Extractable validation of these solutions may involve passing a model solvent system through the filter or soaking the filter followed by examination of the solvent system for filter components.

Flushing filters prior to use may be used to decrease the level of extractables present. If it is determined that flushing is necessary to limit extractables present in the product, validation should be conducted to establish the quantity of product that should be treated, by what volume of fluid, flushed over what interval of time, filtered and discarded, in order to achieve acceptable extractable levels in the filtrate. The ratios of product-treated to volume flushed at what rate, and totally discarded should be reflected in the standard operating procedure used for product manufacture.

Extractable Test

In addition to the product bacteria challenge test, assays of extractable or leachable substances have to be performed. Previous reliance on non-volatile residue testing as a method of investigating extractable levels is discounted by the regulators as being too

insensitive. Extractable/leachable analyses on membranes and other filter components are routinely done by appropriate separation and detection methodologies. Extractable measurements and the resulting data for their individual filter types are usually available from filter manufacturers (Reif, 1998). These tests are performed using specific solvents such as, ethanol, or water at "worst case" conditions. Such conditions do not represent true processing realities. Therefore, depending on the process conditions and the solvents used, explicit extractable tests have to be performed as well. Formerly, these tests were done only with selected solvents, but not with the drug preparations themselves. There was concern lest the drug product's residues mask or interfere with the extractables undergoing measurement (Stone et al., 1994). Recent findings indicate the possibility of evaluating extractables utilizing the actual drug product as the extraction medium.

Such tests are conducted by the validation services of the filter manufacturers using such sophisticated separation and detection methodologies as GC-MS, FTIR, RP-HPLC, UV-VIS, GPC-RI, HPCE and SF. These analytical techniques are employed in order to identify and quantify the individual components extracted from the filter. Their identities are required to judge the seriousness of their presence in the pharmaceutical preparation. Elaborate studies on sterilizing grade filters, performed by filter manufacturers show that there is not a release of high quantities of extractables. The range is from ppb to a maximum of ppm per 10 inch cartridge element; nor have toxic substances been found (Reif et al., 1996; Reif, 1998).

MODEL ORGANISMS

The centerpiece of the subject validation is the assaying of the filter's efficiency in removing organisms from their liquid suspensions. The microbes whose removal from the drug preparation is the object of the filtration should be identified beforehand by microbiological analyses. The bioburden varies in its composition within the pharmaceutical experience. It would be helpful, for reasons of practicality, if the different organism types found to be present would respond uniformly to the filters. To the extent that this is so, the use of model organisms may be relied upon. The bacterium usually selected for challenging the "sterilizing grade" membranes rated 0.2-/0.22-μm-rating is *B. diminuta* ATCC 19146. While useful in its intent, this practice is not universally justified. In particular, the sieve retention mechanism is not applicable to those organisms that undergo size diminutions or shape alterations that permit their penetration of the 0.2-/0.22-μm-rated "sterilizing" membranes. Other organisms are used in the testing of membranes of different sizes or characteristics, for example, *Serratia marcescens* in the case of 0.45-μm-rated membranes.

Conclusions cannot be made regarding the sterile filtration of microorganisms unless methods of quantifying them by culturing and counting are available. Organisms such as the L-forms, nanobacteria, and "viable but non-culturable" entities may not be amenable to such analyses. Concerns about their presence may be justified, but absent the means to cultivate and count them, it is impossible to attest to their complete absence; obviously as determined within the limits of the assay. It follows that a sterilizing filter can be judged only by its performance in the removal of identifiable and culturable organisms known to be present in the drug preparation (Agalloco, 1998).

Use of Isolates

An advantage is seen in the use of isolates obtained from the drug preparation itself. The use of organisms native to the drug composition lessens the artificiality of

employing cultured *B. diminuta* as the test organism. The assay's endpoint involves culturing, growing, and counting the *live* organisms found present in the filtrate. If dead organisms were to penetrate the filter, they would not be detected in the effluent. An absence of live organisms will be taken to mean that the filter completely removed the challenging microbes; that the filter performed its sterilizing function. It is necessary, therefore, to make sure that the absence of live organisms is due to filtration sterilization and not to their being killed by the drug preparation. The very origin of the bioburden organisms shows that the isolates are able to survive in the process stream. This consideration in itself favors the use of process isolates; the concern need then not be entertained that the suspending liquid might be cidal. The possibility of erroneously concluding from the absence of live organisms in the effluent that the filter had restrained the passage of organisms when in actuality they had been killed by the drug preparation is eliminated.

It might have been thought that *B. diminuta* being killed by the drug would render moot the question of its filtrative removal. However, in its role as a model organism for assaying the filtration, it is required also to represent those bacterial types that might not be killed by the drug preparation. When *B. diminuta* are added to the drug solution, care is taken to determine their survival. If they are killed by the drug, the cidal component is removed, or other effective steps are taken, as will be described, to substitute a placebo compounded to mimic the properties of the drug preparation as closely as possible, but without its cidal effect. The absence of the live organisms from the effluent could then properly be credited to their filtrative sterilization.

Size and Shape

If the bioburden isolates can be cultured in the product to a level necessary to provide a challenge of at least 1×10^7 per cm^2 then their physiological state and size characteristics will be the same as in the process stream. If it is necessary to grow the challenge organisms in other than product, it still may be possible to allow them to equilibrate for several doubling times in the product solution so that they will still have the same physiological and size characteristics as organisms cultured in product.

The size of the test organisms is obviously important. It is, therefore, necessary to demonstrate that the bioburden organisms are of appropriate size. That the organisms challenging the test membrane, whose pores are 0.2-/0.22-μm-rated, are neither individually too large nor in aggregated form is assured by their passage through a 0.45-μm-micron rated filter.

It is also necessary to culture the organisms as a monodispersed suspension in numbers adequate to challenge the test and control filters without the complications of filter cake buildup; such as may impose their particle retentions on the operation. Filters of large enough areas are obviously required to meet the challenge stipulation of 1×10^7 (or greater) colony forming units (CFU) per cm^2 of EFA. In the ideal, no pore would escape challenge, and none would be confronted by more than one microbe. The numbers of pores constituting a filter is, in any case, not known. But even if the numbers of pores and organisms could be matched, the laws of probability via the Poisson distribution (Juran, 1974) show that an attempt to lay down an average of two microbes per square micrometer of area will result in 15% of the squares being vacant while another 15% will contain three or more organisms (Johnston and Meltzer, 1979).

Culturing

The challenge organisms can be obtained by introducing their sample into a proper volume of nutrient medium for the period of time suitable for their growth; to the point where they can be counted. The suitability of the growth medium must match the requirements of the particular microbial type(s); these may differ significantly for the various microbes. There is no growth medium that will serve all organism types. As said above, certain types are viable but not culturable. Their presence cannot be assessed by this technique. As already stated, it is only live organisms that can be cultivated to the point where they can be counted. Dead or living organisms that will not develop on the selected growth medium will escape detection. Therefore, of the various types of microbes that may be present, only those for which a suitable growth medium is available can be tested for. The inability to detect an organism type other than the organisms of interest reveals nothing concerning their presence or absence. The count of zero has significance only for the identifiable organisms. The sterility of the effluent is judged only as regards that particular organism type (Agalloco, 1990).

BACTERIA CHALLENGE TEST

The choice of suspending fluid is important. If possible, the best choice is to suspend the challenge organisms in the product solution. This is the method is cited by the 2004 FDA Aseptic Processing Guidance as being preferred. However, in many cases, this is not possible, generally because the product is antagonistic to the challenge organism.

Before performing a product bacteria challenge test, it has to be assured that the liquid product does not have any detrimental, bactericidal, or bacteriostatic, effects on the challenge organisms, commonly *B. diminuta*, being suspended in it. This is necessitated because the microbial assaying is by the counting of live organisms. The assessment is done utilizing viability tests. The product is considered non-bactericidal, if, over the exposure time the viable organism count decreases by less than or equal to 1 log. The organism is inoculated into the product to be filtered at a certain bioburden level. At specified times, defined by the actual filtration process, the log value of this bioburden is tested. If the bioburden is reduced due to antagonistic fluid properties, different bacteria challenge test modes become applicable.

Filtration conditions (i.e. time, throughput, volume per unit filtration area, differential pressure, pressure cycling, flow rate, hydraulic shock) used for validation experiments should mimic, to the extent possible, "worst-case" processing conditions. These are the situations wherein the possibilities of removing organisms are minimal. "Worst case" processing conditions include high pressure, longer filtration time, and greatest volume filtered, and may include other parameters. When considering filtration conditions, as with the suspending fluid, concessions may be required in order to ensure challenge organism viability.

There are three bacteria challenge methodologies designed to confront the filter with the live organisms. They are described within the PDA Technical Report No. 26: Higher organism challenges; placebo (modified product) challenge; and product recirculation through the filter followed by an organism challenge after recirculation. If the mortality rate is low, the challenge test will be performed with a proportionally higher bioburden, designed to permit the challenge level to decline to, but not below, 10^7 per cm^2 by the end of the processing time. In some cases it is possible to modify the product (i.e. removal of preservative or other bactericidal component) to allow for the

survival of the organisms in a product surrogate. If the mortality rate is too high, the common definition of which is >1 log during processing time, a placebo is fashioned by removing the toxic substance, or by modifying the filtration conditions, or product properties to the point where the challenge organisms are not adversely affected. Examples of possible modifications are pH, temperature, etc. If the organism can survive in the product for a period less than the entire filtration time, it may also be possible to shorten the exposure of the challenge organism. This could be accomplished by adding the challenge organisms after the product has been circulated through the test and control filters for a portion of the recirculation time.

In some cases the only option left may be to circulate the product solution through the product filters for the processing time under "worst case" conditions, rinse the test and control filters, and challenge the filters with the challenge organism suspended in saline-lactose broth. However, this would not assess the effect of the product on the challenge organisms. In addition, the interplay of the organisms, product solution, and the filter would remain unexamined. Although this method is not to be preferred, it still provides an indication of what can be expected from the filter's performance during product sterilization.

Afterwards the filter is flushed extensively with water, and the challenge test, as described in ASTM F838-38, is performed. The rinse is followed by the bacterial challenge in saline lactose broth or other non-bactericidal carrier.

During filter validation studies, the entire filtrate must be assayed for the presence of the challenge organism. This is generally accomplished by a second assay filter placed in-line downstream of the test and control filters. This raises anew the question of whether 0.2-/0.22- or 0.45-μm-rated membranes are the more suitable as assay filters. Originally, the rated pore size used was 0.45 μm. The 0.2-/0.22-μm-rated size came to be used by some because it was thought more likely to retain smaller entities more reliably. Present practices, based on several if equivocal studies, sanction the use of either pore size rating.

Filtration conditions, that is, high differential pressures, longer filtration times, and throughputs, that is, greater volume filtered, along with volume per unit filtration area, flow rate, hydraulic shock, and pulsation studies used for validation experiments should mimic, to the extent possible, "worst-case" processing conditions. These are the situations wherein the possibilities of removing the organisms are minimal. "Worst case" processing conditions may include other parameters. When considering filtration conditions, as also the composition of the suspending fluid, concessions may be required in order to ensure a proper organism challenge.

Sampling

The proper management of the sampling technique is itself an important subject deserving of a full but separate discussion. The size of the effluent sample or the number of samples that are analyzed should be large enough to offset the heterogeneity that is typical of suspensions, and to yield a count of some reliability to be made. The suspended organisms will over time adsorb to the surfaces of the sample-container regardless of its material of construction. The resulting CFU counts will be diminished thereby. If the analysis is not to be performed promptly, refrigerated storage may be utilized to prevent or minimize organism growth to the point where die-off or growth alters the original numbers.

The size of an organism changes as it develops through its growth stages during its cultivation. Typically, the growth curve for most organisms placed in a new environment

extends through three phases. During the first or lag phase the organism adjusts to its new surroundings. There is an increase in the individual cell size before there is an increase in numbers. In the second or exponential growth or log phase, attached cells begin to divide, and a logarithmic increase in cells occurs. The cell numbers increase more rapidly than does the cell mass. Thus the cell numbers are increasing, but the size of the individual cells is decreasing. This results from the decreasing food supply, and the accumulation of toxic waste products. There follows the third or maximum stationary phase wherein the number of new cells equals the number that are dying. In the later stages of this phase there are increasing amounts of dead organisms and cell debris. The early stages of the stationary phase are, therefore, better for the detection of the live organisms. Where the exponential growth curve enters the stationary phase, the early beginning of the growth curve, is where the exponential growth curve enters the stationary phase, the early beginning of the growth curve, is where the organism selection is best made.

Organism Counting

The organisms in the sample can be detected and/or counted in a number of ways. A liquid aliquot can be added to a suitable growth medium in liquid form. Following incubation at a temperature and for a time proper for the organism type of interest, the presence of organisms will be made apparent by a clouding of the mixture. When the selected assay involves the isolation of the sample's organisms by filtration, the filter cum organisms are placed on a nutrient medium for appropriately cultivation by incubation for the correct time and temperature. This technique requires aliquot samples large enough to furnish enough organisms for an accurate count to be made. The count should be from 20 to 100 CFU. The number is considered accurate, to plus or minus one-half log by some; others set the limits as plus or minus one log. A CFU reading of 20 would by the latter reckoning represent a value from zero to 100 or 150 CFU. To attain a confidence level of 95% some 22 samples would be needed. When records are kept of successive assays, the trending of the data over time impart to the results a significance that single readings do not have. Nevertheless, in individual tests the CFU counts of 20 or less are usually treated as if they were reliable in their quantification. Thus, assays reading zero organisms are, in effect, taken to signify sterility.

"Sterilizing Grade" Membrane

If the microbiological assay indicates that none of the challenging organisms escaped capture by the filter, it is designated as being a sterilizing filter. Actually, this conclusion is too general in its assumptions. That the filter performed as desired under given circumstances does not ensure that it will necessarily act similarly with other types of organisms, or even with the same organism under different filtration conditions. The earlier belief that sterility resulted exclusively from the particles being too large to negotiate the filter's pores proved simplistic. Each filtration is an individual expression of several factors whose balanced influences govern the outcome of the organism/filter interaction.

"Sterilizing grade" filters are characterized by a bacteria challenge test carried out by the filter manufacturer. This test is performed under strict parameters using a specified growth medium, etc. (ASTM F838-83). This filter manufacturer's organism testing defines the "sterilizing filter" in terms of its integrity test value correlating with a sufficiency of organism retention, namely, 10^7 CFU/cm^2 of EFA. This qualifies it for use

in actual process filtrations. However, "sterilizing grade" filters are not necessarily capable of yielding sterilized product under all circumstances. The process parameters, fluid properties, the polymeric structure of the filter, and the nature of the bioburden will influence the outcome. Consequently, FDA requires validation, documented experimental evidence that the "sterilizing grade" filter does indeed yield a sterile effluent when conducted within stipulated process parameters with the actual drug product and actual bioburden (Jornitz, 2002).

Need for Process Validation

A filter characterized as qualifying for the "sterilizing grade" designation bestowed upon 0.2-/0.22-μm-rated membranes, as just discussed, may not fulfill that role in an actual processing operation. There are many reasons why this should be, given the numerous influences that impact the attainment of sterile effluent. For example, if the test organisms known to be smaller than *B. diminuta*, a tighter filter may be indicated (Kawamura et al., 1998). There are also several other reasons for requiring product bacteria challenge testing. First of all, the influence of the product and process parameters on the viability of the microorganism has to be determined. There may be cases of shrinkage in the size of the organisms due to a higher osmolarity of the product, or to prolonged processing times, or to starvation due to the extremely low assimilable carbon content of the suspending fluid. Secondly, the filter's compatibility with the product and process parameters has to be tested; it should not be assumed. The filter should not show any sign of degradation due to the product. Additionally, assurance is required that the filter will withstand the process parameters, especially the applied differential pressure. For example, pressure pulses, as from filling machines, should not influence the organisms' retention.

It should be remembered that there are two main separation mechanisms involved in liquid filtrations, namely, sieve retention and adsorptive sequestration. In sieve retention, when the smallest particle or organism is retained even by the largest pore within the membrane structure, the contaminant will be removed regardless of the process parameters. This would be the ideal situation of an absolute filtration. Retention by adsorptive sequestration, however, depends on the filtration conditions. Contaminating organisms smaller than the actual pore size can penetrate the filter, but may be retained by adsorptive attachments to the pore surfaces. This effect is enhanced by using highly adsorptive polymers for filter materials; for example, glass fiber as a pre-filter, or polyamide as a membrane in protein processing. Certain liquid properties can, however, minimize adsorptive effects. Borderline compatibilities may enlarge pores, and the fluid's physiochemical properties may alter organism shapes and sizes. Any of these several situations may result in the penetration of the filter by organisms. Whether the fluid has properties that will lower the occurrence of adsorptive sequestration, and possibly allow penetration has to be evaluated using the specific product and specified conditions in the bacteria challenge tests (Jornitz et al., 2002).

Sterilization of Filters

To produce sterile effluent, it seems self-evident that the filter should itself be in a sterilized condition prior to its use. Pre-use sterilization of the sterilizing filter ensures that the filter medium is itself sterile and will not introduce organisms into the process stream. Filters are often sterilized prior to use, either by in-line steamed-in-place (SIP), or by steam autoclaving followed by aseptical installation.

Filters may also be sterilized by gamma irradiation. Less frequently they may be sterilized using ethylene oxide. It is important that the filter membrane and support material remain undamaged under the conditions employed for sterilization. (PDA Technical Report 26). Validation is necessitated for any method of sterilization that is utilized.

With regard to moist heat sterilizations, such as by steam, thermocouples and thermal-resistant biological indicators (BIs) are used. Generally, *Geobacillus stearothermophilus* spores are employed for the validation experiments. Empty-chamber runs are performed to identify and map the slowest-to-heat locations in the autoclave. If the filter is SIP, care must be taken to eliminate any opportunity for condensate to collect, and for areas of entrapped air to be present in the lines or in the filter housings. The presence of water limits the temperature to 100°C, the equilibrium point of water in its liquid and vapor states. Unlike steam, entrapped air cannot impart a heat of condensation to the process. It could, on the contrary, act as an insulator. Heat penetration studies should be conducted to identify the slowest-to-heat locations even in the piping; these studies should also include the monitoring of any location that may be prone to collect condensate. During BI studies, the thermocouples and BIs are placed in the previously determined slowest-to-heat locations during the moist heat sterilization validation. These can provide evidence that the desired temperature was attained even at the sites most likely to be at lower temperatures. If possible, spores can be inoculated directly onto the filter and the filter placed in microbiological culture medium to investigate whether the filter membrane is sterile. Inactivation of the heat resistant spores and adequate heat penetration attest to the sterilization of the filter by the process sterilization cycle.

The radiation dose necessary for sterilization may be calculated using the bioburden present on the filters to be sterilized according to (ANSI/AAMI/ISO 11137, 2006) criteria. Radiation exposure is measured using dosimetry. Biological indicator and/or dosimeters are placed in locations throughout the carrier including sites that are "difficult to penetrate". Following exposure the dosimeters and indicators are removed and examined to determine the adequacy of exposure throughout the carriers.

That a particular filter can serve as a sterilizing filter is established by confronting it with a meaningful organism challenge. The microbiological assessment of whether any of the challenge organisms escaped capture is made by examining the effluent for their presence. The FDA has defined a proper challenge as one that confronts every square centimeter of the EFA with 1×10^7 CFU of *B. diminuta* ATCC-19146.

INGREDIENT AND PRODUCT ADSORPTION

Filter and filtration validations may also address concerns that are independent of the matter of sterility, but that are important to the filtration process nonetheless. A particularly unwelcome tendency on the part of filters, depending upon their porosity and its disposition as pores of various lengths, widths, and tortuosities, is the volume of product they retain following the termination of a filtration. This can be an economic concern especially when expensive biopharmaceuticals are being processed. This volume of liquid can be freed from the pores by applied pressures larger than the bubble points of the several pores. However, the recovered liquid should be segregated from the filtered product. It may contain organisms released by the filter under the impetus of the pressure used in expelling the liquid. If not inappropriate, it should be combined and processed with the next batch of product of similar composition.

It was earlier stated that electrical charge phenomena were involved in the adsorptive interactions between filters and particles, such as organisms, suspended in the liquid vehicles. Likewise, the filters in response to exactly the same type of electrical forces can adsorb molecules from the solutions they process. As a result, the filter medium may adsorb and remove some amounts of certain components of the drug preparation undergoing filtration. Indeed, the active drug ingredient may itself be affected. This could result in some portion of the product batch containing a diminished level of active drug. Some filter polymers are more inclined to specific adsorptions than are others. Perhaps more pointedly, the molecular structure of certain ingredients containing charged or partially-charged atoms will more avidly bond with the drug molecules that are categorized by their own partial-charges.

Validation of the filter medium intended to filtratively sterilize a drug product, therefore, includes testing to determine whether the active ingredient is adsorbed by the filter medium. Generally this is accomplished by passing the drug product solution, with its known concentration of the active ingredient(s), through the selected filter. The filtered material is then assayed for the level of active component present in the filtrate. A decreased level of active ingredient in the filtrate is indicative of the ingredient being adsorbed by the filter medium. Depending upon their polymeric nature, filter membranes can, in addition to active ingredients, adsorb, for example, preservatives such as benzalkonium chloride or chlorhexadine during a filtration of a product containing them. Likewise, the non-specific adsorption of proteins by filters is an important matter. It is customary for testing of this type to be performed using some three filter samples, each from a different lot of filters.

Often, the filter will become quenched; that is, the adsorptive sites on the filter surface will presumably become saturated, and the adsorptive removal of the ingredient will slow and ultimately cease. Testing should be performed to determine whether this does indeed occur after some volume has been filtered. If this is the case, then studies can be used to determine the volume of the filtrate to be discarded prior to filling the remaining portion of the product batch.

Such adsorptive losses from the product formulation can be slowed or avoided by saturating the membrane with the preservative, or with serum albumin is in advance of the filtration. Preservative loss, particularly when the packaged product in repeatedly revisited in multi-use applications such as with contact lens solutions, can be dangerous. Long-term use, and the possibilities for organism growth in the absence of sufficient preservative can be a serious matter (Udani, 1978; Chiori et al., 1965).

Similarly, problematical would be the adsorptive removal of proteins from a biological solution. To ascertain the correct dosage of a protein preparation undergoing filtration, adsorption studies may have to be performed to define the most suitable filter in terms of its polymeric identity, and construction. In addition, determining the pre-rinsing procedure, and flow conditions necessary to its use should be included. Any yield losses caused by non-specific adsorptions can be costly with respect to lost product and its market value. Adsorption studies can be helpful in optimizing downstream processes with regard to yield loss. Yield losses may also impact upon capacity problems, which are existent within the biotech industry. Yield losses have a detrimental influence. Most commonly, such losses can also be attributed to non-specific adsorptions caused by the wrong choice of membrane polymer. Proteins adsorb avidly to filters composed of nitrocellulose, including the mixed cellulose esters, probably by hydrogen bonding. They adsorb strongly also to filters composed of polyamide polymers. It is understood that proteins undergo hydrophobic adsorptions. These are peculiar to filters of non-polar polymeric structures. Thus, the low adsorption of proteins exhibited by the cellulose acetate filters.

Particulate Matter

Particulates are critical in sterile filtration, specifically of injectables. The USP 27 (2004) (United States Pharmacopoeia) (2004) and BP (British Pharmacopoeia) quote specific limits of particulate level contaminations of defined particle sizes. These limits, being regulatory requirements, must be met. Therefore, particle release from sterilizing grade filters necessitates measurement. Filters are routinely tested. Particle enumeration is performed with laser particle counters. Such tests are also performed on the actual product to prove that the filtration step, especially under process conditions, did not result in an increased level of particulates within the filtrate. Specific pre-use flushing protocols, if necessary, can be established for the filters being used, to flush out loose particulate debris. The flushes, singly or repetitive, whether by water, alcohol, or some other suitable liquid would be defined in terms of volume and rate of flow. Each flush would be followed by a particle count on the filter effluent to determine whether the particles still being shed had attained an acceptable level. These tests are also applicable to prefilters to reduce the possibility of particulate contamination generated within the process steps. USP standards for large-volume parenterals (LVPs) stipulate they are not to contain more than 5 particles per mL of sizes greater than 25 μm, and not more than 50 per mL 10 μm or larger. This equates to 50,000 of 10 μm or larger, and 5000 of 25 μm or larger per liter. In addition, no visible particles (haze) are permitted (USP 27). For small-volume parenterals (SVPs) intended for intravenous and certain other injections, the particulate level is confined to one-fifth the LVP limit on a dosage basis, regardless of its volume. This amounts to 10,000 particles of 10 μm or larger, and 1000 of 25 μm or larger per dose. LVPs are preparations delivered through the skin in volumes larger than 100 and up to 1000 mL. They include irrigation solutions and parenteral dialysis fluids which may be used in 1.5–2 L quantities. Also included are flexible pouches for blood collection to which 5 mL anticoagulant have been added under LVP operating conditions. SVPs are preparations below 100 mL in quantity. They are often administered in a piggyback arrangement by way of the LVP delivery set.

Filter Integrity Testing

So obvious are the outcomes of filtration exercises dependence upon the integrity of the filters that are employed, that calling attention to their integrity testing requirements seems superfluous. Post-use integrity testing of product sterilizing filters is a regulatory requirement. It represents the proof of the pudding, as it were. Its application and proper performance is indispensable to the entire picture of sterilizing filtrations. Pre-use, post-sterilization integrity testing of sterilizing filters, on the other hand, although strongly urged by the regulations, is often overlooked by the regulatory authorities. The pre-use, post- sterilization demonstrates that the filter is properly installed and that the filter was not damaged during the sterilization process. However, its manual performance necessitates an invasion of the filter train downstream of the filter. This presents an important risk to the system's asepsis. Increasingly, its omission is overlooked. Neglecting this particular testing represents a business risk to the drug preparer, but not a physiological risk to any drug recipient, because a compromised filter will be discovered after the batch is processed. Reworking or discarding the processed batch will then be required. Clearly, the integrity testing of sterilizing filters prior to use, but after installation, obliges that the testing method not involve breaking the sterile line downstream of the filter. If this cannot be accomplished, then pre-use integrity testing

should not be performed. This can be managed by use of an automated integrity test machine; such perform the testing from the upstream side of the filter.

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14

Extractables and Leachables Evaluations for Filters

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INTRODUCTION

Sterile filtration is one example of a process using a polymeric component for which extractables and leachables are a concern. Ideally sterile filtration removes unwanted particles and bacteria while allowing the formulation to remain unadulterated. Although hundreds of liters of pharmaceutical formulation may be filtered through just a few square meters of filtration area, the effect of leachables cannot be underestimated due to the intimate interaction of the formulation with the polymer. While the filtration area may be small, the surfaces that experience product contact are several thousand times larger due to the pore structure of the polymeric filter materials. In Figure 1, the top surface is the reported filter surface area. However, the solution contact surface also includes the surfaces of the porous "sponge" structures within the filter. Leachables may also come from filter housings, membrane support layers, O-rings, or any other polymeric components of the filter. Identifying and quantifying these contaminants can be an analytical, albeit necessary, challenge. The necessity is more apparent in the final steps of downstream processing, where impurities may have a greater effect on the product.

The evaluation of filters for leachables and extractables is both a regulatory requirement and an appropriate safety concern. A filter incompatibility may be identified by leachable and extractable testing even when other filter validation methods, such as bacterial challenge testing, show acceptable results.

Regardless whether driven by regulatory or safety concerns, it is the responsibility of the drug manufacturer, not the filter vendor, to show a filter is compatible with the manufacturing process stream and does not add levels of contaminants that could alter the safety, identity strength, quality, or purity of the drug product.

DEFINITIONS

The terms extractables and leachables are often used interchangeably within the literature and regulatory guidances. However, in recent years, there has been an effort to standardize the terminology to differentiate leachables from extractables.

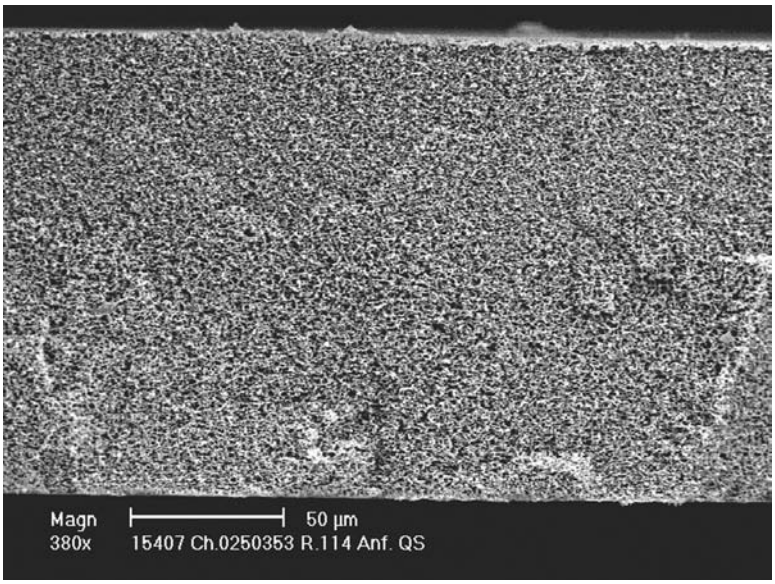


FIGURE 1 Cross section of a polyethersulfone 0.2 µm filter. *Source*: Courtesy of Sartorius AG.

Extractables

Extractables are defined as compounds that have the potential to be removed from a contact material by use of exaggerated extraction conditions. Extractables are usually detected by extracting a material with multiple solvents of varying polarity at elevated temperature. The temperature should be sufficient to enhance the migration of compounds out of the materials but not so high as to cause the material to become unstable, that is, it should not approach the glass transition point in the case of a polymer.

Leachables

Leachables are compounds that actually do migrate out of a contact material into the actual pharmaceutical formulation during normal use conditions with respect to key operating parameters including but not limited to time, temperature, pH and any filter preparation steps such as sterilization and rinsing. Leachables are typically a subset of extractables.

SOURCES OF EXTRACTABLES AND LEACHABLES IN FILTERS

Extractables and leachables can come from any material, for example, plastic, glass or metal. However, they are most often associated with the use of elastomers and plastics. The evaluation of extractables and leachables has been well established in the pharmaceutical field when considering the use of plastics and rubber stoppers in container-closures as indicated by the guidance published in 1999 by the FDA for container closure systems (CDER, 1999). The discussion of extractables and leachables in pharmaceutical processing materials such as filters has become more acute as single use disposable materials have become more common.

Polymer-based plastic materials would be difficult to process and unstable without the use of additives. Most polymeric additives are not covalently bonded to the polymer.

Therefore, they can migrate from the polymer to a contacting solution. Their actual migration depends on the nature of the contacting solution and the conditions during contact. Fortunately, most pharmaceutical formulations are aqueous-based and most polymers and their additives are, by nature, organic and hydrophobic. Consequently, polymeric components and additives do not readily migrate into water-based pharmaceutical formulations.

That does not mean polymeric additives cannot leach into an aqueous pharmaceutical formulation. While water for injection (WFI) may not be expected to leach very much out of a hydrophobic polymer, pharmaceutical formulations can have significantly different properties from WFI. One example of this is the use of solubilizing agents such as surfactants which enhance the solubility of a pharmaceutical product. Biopharmaceuticals typically have more organic ingredients with the potential to leach compounds out of plastics.

Antioxidants

Plastics made of polymers that contain C–H, C–OH, or C=O bonds are subject to oxidation. This is observed daily in the burning of fossil fuels to create energy. As a result, most pure polymers quickly degrade through oxidation. Polymers without these bonds, such as polytetrafluoroethylene, are the most resistant to oxidation. To reduce oxidation, polymers require the use of antioxidants. The most common type are phenolic antioxidants such as 2,4 di-t-butylphenol and hindered phenolics such as found in the Irgonox, Ethanox, and Lowinox product lines. The phenolic rings of the antioxidants capture free radicals before the free radicals oxidize the polymer which would cause additional polymer breakage.

Lubricants

Polymer additives can include internal lubricants (to lubricate the polymer chains during processing) and external lubricants (to lubricate interface between the polymer and the processing equipment during processing). Examples of lubricants are silicone and fatty acids such as stearic acid.

Oligomers and Monomers

Oligomers and monomers of the base polymer can be the result of incomplete polymerization or they can be the degradation product of oxidation of the fully formed polymer.

Plasticizers

Plasticizers, a common additive in polymers such as polyvinylchloride, to enhance flexibility, are not common in filters. However, compounds such as di-2-ethylhexylphthalate are known to be associated with extractables from polyolefins (Jenke et al., 2005).

Wetting Agents

Wetting agents are compounds more common to filters than to other disposable polymeric components used in pharmaceutical processing. Many of the filter materials

such as polyvinylidine fluoride and polyethersulfone are naturally hydrophobic. In order to make the filter hydrophilic, wetting agents are added during the manufacturing process. Wetting agents include polyvinyl pyrrolidone, polyethylene glycol and polyacrylates. They can be coated, impregnated or covalently bonded so as the make the filter surfaces hydrophilic.

REGULATORY REQUIREMENTS

The regulations, globally, for many pursuits lack clarity regarding the specific requirements. Industry must interpret to the best of their ability the actions needed to meet regulatory mandates. The interpretation and implementation activities are similar to solving a puzzle. The requirement to perform extractables and leachables testing on processing materials (including filters) is a perfect example of this puzzle. A few of the extractable/leachable puzzle pieces can be found in published guidances yet even these lack details and specifics needed to develop effective filter extractable/leachable validation programs. The best guidance truly comes from published papers and conferences sponsored by various organizations. With real life examples, industry can find guidance to develop and implement filter extractable/leachable validation programs.

Under US/FDA regulations, there is no doubt filter users must validate filters for extractables and leachables. While FDA regulations and guidances lack clarity for the specific requirements of extractables and leachables testing, FDA Warning Letters (readily accessible on the FDA's website) demonstrate FDA's expectation on the evaluation of filters for extractables and leachables (FDA).

FDA Warning Letter dated August 16, 2005 (following inspection of a pharmaceutical manufacturing facility in Switzerland):

> . . . Further, it is unclear to us whether you have conducted **filter extractable and leachable testing** with product. If you have this data, provide it to us. If not, let us know when you will be able to provide it to us . . ..

FDA Warning Letter dated September 30, 2005 (following inspection of a facility engaged in manufacturing sterile ophthalmic solutions):

> . . . You have failed to validate the [redacted] membrane filter used for filter sterilization for compatibility, *extractables* and microbial retention.

The Center for Drug Evaluation and Research (CDER) published an industry guidance in November 1994 referencing the requirement of extractables evaluation as part of process validation in applications for human drug products: The Guidance for Industry for the Submission Documentation for Sterilization Process Validation in Applications for Human and Veterinary Drug Products (CDER, 1994a) states:

> The specific bulk drug product solution filtration processes, including tandem filter units, prefilters, and bacterial retentive filters, should be described. . . .. Any effects of the filter on the product formulation should be described (e.g., adsorption of preservatives or active drug substance, or extractables).

This guidance describes the requirement for scientifically valid methods performed at conditions "fully representative and descriptive of the procedures and conditions proposed for manufacture of the product."

Similarly, FDA's pharmaceutical Good Manufacturing Practices (GMPs) mandate that materials used in the production of pharmaceutical products be compatible with

the drug products (*Code of Federal Regulations, Food and Drugs* **Title 21, Part 211.65**). Title 21 of the Code of Federal Regulations (CFR) Part 211.65 states:

> Equipment shall be constructed so that surfaces that contact components, in-process materials, or drug products shall not be reactive, additive, or absorptive so as to alter the safety, identity, strength, quality, or purity of the drug product beyond the official or other established requirements.

The requirement for evaluation of extractables and leachables moved industry to begin its quest for clarity and definition in the early 1990s. Most of the early focus was on final container/closure systems but processing materials were also being considered. The Division of Manufacturing and Product Quality at CDER published a Human Drug cGMP Notes memo in September 1994 containing policy questions and answers (CDER, 1994b). One of the policy questions was specifically: "Does a manufacturer need to test each drug product for filter extractables?" While the answer was "no" the discussion in the answer stated:

> This does not mean that the drug manufacturer does not need to have information concerning filter extractables. They must have data showing the identity, quantity and toxicity of the extractables. They should also have the methods and solvent systems used to obtain the amount of extractables per filter.

Since September 1994, the industry and regulating agencies have discussed, reviewed, examined and clarified the expectations for extractables and leachables testing. As mentioned earlier in this chapter, even the respective definitions have evolved over time. At this time there are no specific guidances for extractables and leachables but industry associations are collaborating with regulating agencies to define the standards of practice for this field of inquiry.

CDER published a Guidance for Industry entitled Container Closure Systems for Packaging Human Drugs and Biologics in May 1999 (CDER, 1999). This guidance, while not specifically addressing processing equipment, gives an indication of the types of drug products that the FDA considers to be the highest risk for extractables. In this guidance, the FDA classifies drug products on the basis of the risk of administration route (e.g., injectables, topical, oral) and on the level of intimate contact (solutions, aerosols, powders). A drug that is to be administered as an injectable or inhalant, will have the highest level of regulatory concern. Oral or topical drugs will have lower concern. With regard to extractables testing, it states:

> When feasible, the preferred solvent (for extractables testing) would be the drug product or a placebo vehicle.

The Parenteral Drug Association (PDA) published Technical Report 26 "Sterilization Filtration of Liquids" to guide users with the selection and validation of sterilizing-grade filters (PDA, 1998). Section 4.4 of the report addresses extractables and specifies:

> It is the user's responsibility to demonstrate that the product does not contain objectionable levels of extractables from the filter.

> The filter user is responsible for obtaining extractable data for the drug product formulation.

Outside of US regulations, Health Canada also speaks to the filter requirements in a Draft Guidance for Industry (**ANDSs, 2001**). In the Process Validation section it states:

> Filters used should be validated with respect to pore size, compatibility with the product, absence of extractables and lack of adsorption of the drug substance or any of the components.

The Canadian regulatory basis again comes from the GMPs. Part C, Division 2, in the Equipment section, C.02.005, states:

> The equipment with which a lot or batch of a drug is fabricated, packaged/labeled or tested shall be designed, constructed, maintained, operated and arranged in a manner that
>
> a. permits the effective cleaning of its surfaces;
> b. prevents the contamination of the drug and the addition of extraneous material to the drug; and
> c. permits it to function in accordance with its intended use.

The pharmaceutical industry in Europe faces the same challenging puzzle when it comes to finding clear guidance on filter validation for extractables and leachables. In the rules governing medicinal products (in the European Union), Volume 4 titled Good Manufacturing Practices, Chapter 3, paragraph 3.39 has a statement very similar to that in US/FDA and Canadian Food and Drug regulations:

> Production equipment should not present any hazard to the products. The parts of the production equipment that come into contact with the product must not be reactive, additive or absorptive to such an extent that it will affect the quality of the product and thus present any hazard.

As is the case in the US, the European Union has better defined requirements related to packaging materials (final container/closure systems) and some guidance can be found by reviewing these documents. In 2004 the European Agency for the Evaluation of Medicinal Products (EMEA) published the Guideline on Plastic Primary Packaging Materials (**EMEA, 2004**). This guideline used in conjunction with several Committee for Proprietary Medicinal Products (CPMP) guidelines provides some basis for extractables/leachables testing (e.g., Notes for Guidance on Development Pharmaceutics (CPMP/QWP/155/96); Stability Testing: Stability Testing of New Drug Substances and Products (CPMP/ICH/2736/99) (Revision of CPMP/ICH/380/95); and Stability Testing: Stability Testing of Existing Active Substances and Related Finished Products (CPMP/QWP/122/02)) for European regulated products.

Clearly, filter users are required to validate their filters for extractables and leachables. It is the user's responsibility to develop and implement programs to meet this requirement. It is recommended that the user include key stakeholders in the planning and implementation process. Being familiar with the FDA's Pharmaceutical Quality Initiative as defined in its Pharmaceutical Quality Assessment System (PQAS) (CDER, 2004; FDA, 2004) is key to developing and implementing an acceptable filter validation program. Incorporating the concepts included therein can lead to a scientifically sound program allowing for increased process and product understanding. Quality by design can be a natural consequence if programs such as filter validation are addressed early in the product lifecycle. Regulating bodies have shown flexibility in how extractables/leachables can be addressed as long as sound scientific principles and innovative problem solving are used. In the end, users who clearly have an overarching goal of better understanding their process and product will likely have the most success.

DEVELOPING AN EXTRACTABLE AND LEACHABLE PROGRAM

Often a process uses many filters including feed stock filters, particulate filters, cross-flow (also referred to as tangential flow) cassettes along with the final sterile filters. It can be a daunting job to decide where to focus an extractable program and create a strategy to effectively implement and meet regulatory and safety requirements.

Product Lifecycle

It is suggested to start early in the product lifecycle. If extractable/leachable results are obtained which present safety and possibly efficacy issues then alternate filters can be considered and evaluated early in the development process. Alternate filters may need to be considered for possible changes later in the development cycle. Other factors in the drug development process may lead to necessary changes in filter types. Having options available could save time in fast-paced development programs.

Create a Team

It is important to identify key stakeholders in the filter validation program. Create a dedicated team of scientists, Quality Assurance, validation specialists, etc., to plan and implement the program.

Filter Risk Assessment

Evaluate the particular regulatory and safety risks of process filters using a risk assessment approach. Consider different criteria that are appropriate to the application. Key risk factors to consider are the following:

1. *Drug Dosage Form*: The safety risk of a drug depends on the route of administration and the size of the dosage. Routes that allow a drug to pass into the blood stream more easily will be subjected to more scrutiny with regard to impurities. In general, the FDA Guidance on Container Closures (CDER, 1999) indicates the level of concern is:
   Inhaled Drugs > Parenteral Drugs and Ophthalmic Drugs > Topically Applied Drugs > Oral Drugs.
   In addition, the dosage of the drug will be factored into the regulatory and safety concern. A drug product that is administered in large quantities on a regular basis will have higher concern than a drug product that is only occasionally administered in a small quantity.
2. *Location of the Filter in the Process*: The closer a filter is to the finished product, the higher the safety and regulatory concern. The final sterile filtration will have the highest concern because any leachables will be contained in the final product. Filters further upstream will be less of a concern. Process steps that allow for impurities to be removed will lower the concern. An example is the use of cross flow filters for diafiltration to remove known impurities. Often the diafiltration volume is many times larger than the retained fraction which gives solubilized leachables an opportunity to be flushed from the system. However, one should be prepared to address whether the leachables would rather partition into the retained portion because of the chemical nature of the drug product.

3. *Composition of Filtration Stream*: Since filters are usually made of organic polymers, process streams with high organic content tend to enhance leaching. A process stream that is organic or contains high proportions of an alcohol such as ethanol, will be more of a safety and regulatory concern.
4. *Contact Time*: While filters generally have fairly short contact time compared to final container/closures or in-process storage materials, some filters may be used for several days as in the case of a blow/fill/seal operation. Longer contact time will generate a higher level of leachables.
5. *Processing Conditions*: High temperatures and pH extremes can potentially increase the level of leachables and lead to higher safety and regulatory concern. Contact time, i.e., processing time, plays a role in the opportunity of polymeric attack or leachable extraction.
6. *Pretreatment Steps*: Filters are often pretreated prior to use. Typical pretreatments are steam sterilization, gamma irradiation and rinsing.
   a) *Gamma Irradiation*: Increases extractables and leachables. Organic materials can be degraded when exposed to gamma irradiation. The degradation continues over any storage time. Therefore, the shelf life for gamma sterilized goods must to be established.
   b) *Steam Sterilization*: Neutral affect on extractables and leachables. The high temperature during steam sterilization may bring extractables/leachables to the surface of the polymer. However, volatile extractables/leachables may be removed by steam sterilization.
   c) *Rinsing*: Decreases extractables and leachables. Rinsing, either with water or with product, is an effective method to reduce the level of extractables/leachables in a product.

A published example of the risk assessment approach can be found in the December 2002 BioPharm publication. Priority is established by assigning numerical risks to each of the categories to achieve a ranking of filters with the highest degree of concern (Bennen et al., 2002).

Obtain Information from Filter Manufacturers

Extractable data generated by filter manufacturers may prove helpful in planning the extractables/leachables program. This data may identify process parameters and those filters with more propensity to add leachables under the process conditions to the product stream. Additionally, it is important to obtain verification of the materials of construction from filter manufacturers, especially within different sizes of the same filter. Due to the design of many extractable/leachable tests it may be necessary to perform the test with smaller filters than those used in the process. This also facilitates detection because the extractables/leachables are concentrated in the smaller volume of extractant used with the smaller filters.

A capsule should not be substituted for a cartridge because, while the filter material and support structures may be the same for each filter, the filter housing material changes the relative ratio of the materials of construction leading to differing levels of extractables/leachables and possibly even new extractables/leachables if the filter housing introduces a new material.

Results of the risk assessment and the information obtained from the filter manufacturers can be used to create a list of filters requiring validation.

Possibility of Grouping Filters

It is important to consider multiple manufacturing processes at one facility as well as redundancy within one manufacturing process. This allows for the development of grouping strategies. Efficiencies can be incorporated into the program if the same filters are used for similar process streams. Dependent upon the validation strategy employed by the organization, it may be possible to validate one type of filter used for the same type of media or process stream in multiple manufacturing processes.

Grouping Strategy Examples:

1. If the same filter type is used on five different concentrations of NaCl buffer then only the low and high concentration could be tested.
2. If different sizes of the same filter are used for the same process stream, then only one size is tested as long as the materials of construction are identical for the different sizes.
3. If the same product is filtered by the same filter in multiple filtration steps but each step uses different temperatures and contact times then testing the highest temperature and longest time could be acceptable.

The grouping strategy concept can possibly provide advantage by reducing the number of extractables/leachables studies. However, it is important to carefully review the materials of construction and obtain assurance from the filter manufacturer indicating this to be true.

As the extractable program is developed the type of testing to be performed must be determined. Generally, extractables testing is done early in the program, before leachables testing. In some cases, it may be sufficient to do only one or the other. This decision would typically be based on the risk assessment and information obtained from the manufacturer.

EXTRACTABLE TESTS

The goal of extractables testing is to facilitate the extraction of as many compounds as possible under relatively extreme conditions. The goal is not to break down the polymer into individual components to the point of the polymer losing its integrity. Rather, the goal is to subject the polymer to "vigorous" conditions to maximize the number of compounds that can migrate out of the polymer.

Choice of Extractables Solvents

For filters, the extractions are normally performed with at least two solvents. The choice of solvents is dictated by:

1. the intended use of the filter and
2. the materials of construction of the filter.

For filters that are used in aqueous-based systems, the use of high purity water (e.g., WFI) and a low molecular weight alcohol such as ethanol or isopropyl alcohol (IPA) are appropriate.

For filters that are used with organic solutions, extraction with an organic solvent such as hexane is recommended along with a low molecular weight alcohol.

Another consideration is to choose a solvent with properties that make identification easier.

1. Solvents should be easy to work with for identification. For example, octanol is effective at extracting organics from polymers. However, due to certain chemical characteristics of octanol, it is difficult to concentrate the extractables found in the octanol using standard techniques such as evaporation and solid phase extraction.
2. Solvents without significant impurities should be chosen for a cleaner analysis.

Extraction Methods

The extraction of a filter can be performed by one of several methods.

1. Soak with (dynamic) or without (static) agitation. In this case, the filter can be submerged in (if it is a cartridge) or filled with (if a capsule) the chosen extraction solvent(s). Agitation can be achieved by shaking the filter with the extraction solution.
2. Recirculation. The extraction solvent can be recirculated through a filter. This will enhance the extraction because the flow causes a forced convection that minimizes the formation of a concentration gradient that can slow the migration of the extractables.

The effect of extraction technique on the rate and level of extractables obtained was studied in the following experiment.

1. *Extraction Technique Experiment*: Three identical filter capsules were extracted with IPA at room temperature for 48 hours. Two were filled and placed on separate shakers with the ends plugged with glass stoppers. The third filter had IPA recirculated using a dual headed peristaltic pump. The control sample for the filters on the shakers was IPA in a glass flask. The control for the recirculation extraction was the second head of the peristaltic pump with tubing and flask but without the filter. The schematic of each test is shown in Figures 2 and 3.

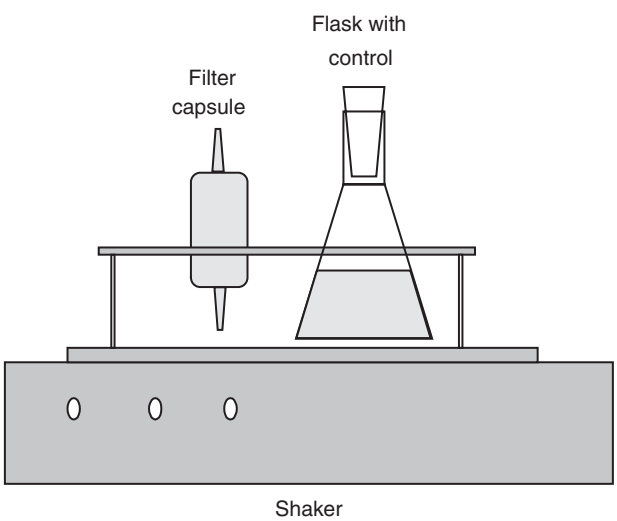


FIGURE 2 Schematic of extractable test with shaking.

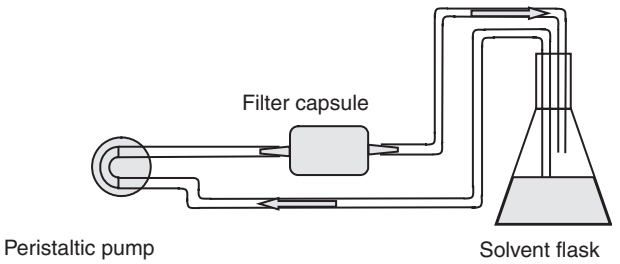


FIGURE 3 Schematic of extractable test using recirculation.

Samples were taken from the shaken filter capsules, the shaken controls and the solvent flasks of the recirculation extraction and control over a 48-hour period. The samples were analyzed by reversed phase-high performance liquid chromatography (RP-HPLC). The level of the extractables was estimated by summing the peak areas of all the extractable peaks.

The extractable peak areas versus time are shown in Figure 4. After 8 hours, the filter capsule shaken at 180 rpm had a higher level of extractables than the filter capsule shaken at 120 rpm. After 24 and 48 hours, there was negligible difference between the two shaken filters.

2. *Extraction Technique Experiment Results and Conclusion*: The filter capsule extracted using recirculation showed lower levels of extractables through the 48 hours. This can be explained because the volume of IPA necessary to fill the capsule, tubing and solvent flask was about double that required to fill the shaken capsule filters. It would be expected the mass of extractables removed from the filter would be higher than using recirculation because of the constant replacement of solvent in the pores of the filter. This is true if the difference in volume is accounted for when comparing the sum of the peak areas. However, the detected concentration of the extractables as indicated by the total peak area is lower for the recirculation because the larger volume dilutes the extracted compounds.

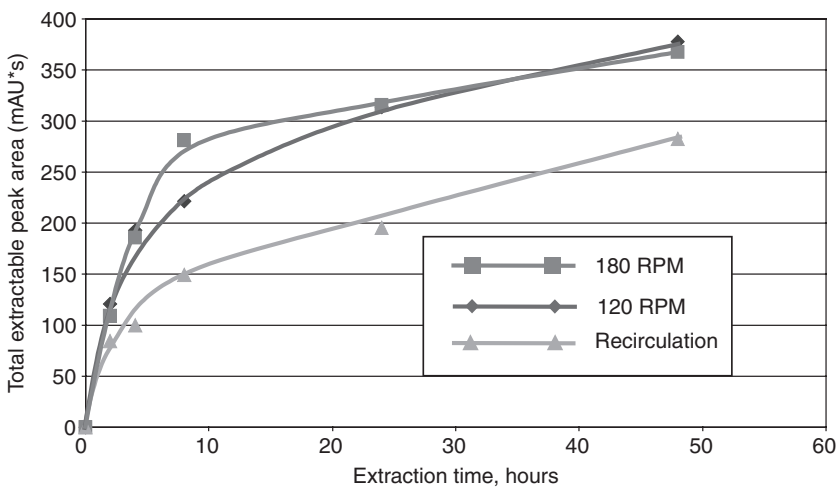


FIGURE 4 HPLC-UV peak area of extractables during a 48-h extraction for shaken filter at 120 and 180 rpm compared to recirculation.

This experiment demonstrated shaking is an acceptable extraction technique. It is vigorous enough to promote extractables in a relatively short period of time. Shaking may actually facilitate increased detection due to the lower volume required.

LEACHABLE TESTS

The goal of leachables testing is to determine what migrates out of the filter into the pharmaceutical formulation when exposed to process conditions that are typical or that test the extreme limits of acceptable conditions. Since leachables are typically a subset of extractables, some filter users perform only leachable tests. Whenever possible, the leachable tests should be performed with the same filter as is used in the process.

The conditions for leachables testing are determined by the maximum acceptable range of the process conditions. The test conditions are then chosen based on worst case conditions. Table 1 shows how this might be done.

Test Parameters and Extraction Conditions:

1. *Filter Size*: In the example shown in Table 1, the manufacturing process uses three 30 inch filters. It is acceptable to test with one 10 inch filter if the materials of construction can be verified to be equivalent in the two sizes. The filter manufacturer can usually provide a letter to this effect upon request.
2. *Filtration/Extraction Product Volume*: If the filter is used to process 2000 L, it is considered advantageous for the extraction to be performed with a substantially smaller volume because the concentration of leachables will be higher with a smaller volume and therefore easier to detect and identify. The process volume and extraction volume cannot be directly compared because the process uses a 30 inch filter and the extraction will be performed with a 10 inch filter. To normalize the difference in filter surface areas, it is necessary to determine the ratio of the extraction volume to the surface area. In this case, testing a 10 inch filter in only 2 L of formulation leads to a volume/surface area ratio approximately 100× that of the process.
3. *Rinsing*: As mentioned previously, rinsing generally decreases the level of leachables. The rinsing volume should be no more than is used for the process

TABLE 1 Comparison of Process Conditions to Leachables Test Conditions

	Process conditions	Test conditions
Filter part number	Part number for 30 inch filter	Part number for 10 inch filter
Filter area	3 filters × 2.1 m^2 = 6.3m^2	0.7 m^2
Product name	API name	API name
Filtration/extraction product volume	2000 L	≤2 L
Product volume/surface area	2000 L/6.3 m^2 = 320 L/m^2	2 L/0.7 m^2 = 2.9 L/m^2
Rinsing	20 L WFI	2 L WFI
Sterilization	125 °C, 30 min	126 ± 1°C, 30 min
Contact time	Up to 4 h	At least 4 h
Temperature range	15–30°C	30 ± 5°C

after adjusting for the size of the filter. To make rinsing "worst-case", the rinsing step can be eliminated or, the rinsing volume can be significantly less than that used for the process. In this case, 2 L of rinse is used for a 10 inch filter compared to 20 L for three 30 inch filters.

4. *Sterilization*: The sterilization step performed in the leachables study should be comparable to the sterilization step used in the process. This is especially true for filters that have been gamma irradiated.
5. *Time*: The length of an extraction should generally be at least as long as the total contact time in the process.
6. *Temperature*: The extraction temperature should be close to the maximum temperature of the process filtration step. It may be acceptable to do an accelerated test at an elevated temperature. However, an accelerated test is subject to validation of the temperature/time correlation.

As with extractables testing, leachables testing can be performed with soaking (static or dynamic) or with recirculation. The control is a sample of the pharmaceutical formulation exposed to the same test conditions as the filter but with no filter contact. For a static or dynamic extraction, the control can be placed in a glass flask sealed with a ground glass stopper (use of rubber stoppers should be avoided). For a recirculation extraction, the control is the exact same recirculation system, ideally operated simultaneous to the filter recirculation. A dual-head peristaltic pump is well-suited for this.

It is not usually necessary to test three lots of filters for leachables. While there can be lot-to-lot variation in the concentration of the individual leachables, it is rare for the number or identity of extractables/leachables to vary between lots. Still, there is no harm in testing three lots and it should be a consideration during the risk assessment phase.

ANALYTICAL METHODOLOGY

Extractables and leachables from filters are generally present at concentrations below 10 ppm and often below 1 ppm. At these low levels, sensitive analytical techniques must be used. In the case of leachables, the formulation components (active pharmaceutical, buffers, and other additives) are present in much greater concentrations than the leachables, thereby increasing the analytical challenges. A publication by Reif et al. (1996) presents the techniques for which extractables analysis from filters is based. In the referenced study, extractions were performed on eight types of filters, methodology was discussed and extractables were identified.

The analytical techniques can be either specific or non-specific. Specific analytical techniques can separate the individual components of the samples. These include liquid and gas chromatography equipped with various detectors (e.g., ultraviolet-visible diode array, mass spectrometer, flame ionization, etc.). Non-specific techniques look at the bulk solution. These include techniques such as total organic carbon measurements (TOC), non-volatile residue measurements (NVR) and fourier transform infrared spectroscopy (FTIR).

Specific Analytical Techniques

Specific analytical techniques such as high performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS) are the standards for extractable

and leachable testing. Both techniques allow for separation of the sample components which is necessary for identification. For leachable testing, specific analytical techniques are necessary to separate the leachables from the components of the formulation. FTIR can be used successfully to facilitate identification of an extractable or leachable once the compound has been separated (isolated) and concentrated from the total sample.

No single analytical technique is capable of detecting all possible extractables and leachables. For this reason, a combination of two analytical techniques is recommended. The use of two techniques is supported in the ICH Q2(R1) guidance (ICH, 2005) on analytical methods. While it is not the goal of this chapter to review analytical chemistry techniques in detail, a brief overview is provided. Most extractables and leachables analyses involve HPLC with UV detection, HPLC with MS detection and GC-MS. Table 2 summarizes the advantages and limitations of the most common analytical techniques.

TABLE 2 Specific Analytical Techniques for Extractables and Leachables Analysis

Method	Description	Advantages	Limitations
HPLC with UV Detection	Compounds are injected into a chromatography column that separates based on polarity of molecule	• High sensitivity ~0.05 ppm • Able to detect wide range of molecular sizes	• Cannot detect molecules without a UV chromaphore such as saturated hydrocarbons
GC-MS	Sample is evaporated and injected into a chromatography column that separates based on polarity, molecular size, and vapor pressure	• High sensitivity ~0.1 ppm • Can detect non-UV active molecules • MS fragments are basis for identification	• Compounds must be at least semi-volatile and thermally stable
LC-MS	An HPLC with mass spectrometer detector in place or in conjunction with a UV detector	• High sensitivity ~0.05 ppm • Can detect non-UV active molecules • Able to detect wide range of molecular sizes • MS fragments are basis for the identification	• Not all molecules can be ionized
FTIR	Infrared energy is passed through a purified compound. Absorbance pattern indicates the presence of specific chemical moieties	• FTIR of a pure compound can be matched with commercial databases to provide identification of compounds	• Minimal capability to analyze mixtures • Limited sensitivity • Can be helpful to identify extractables when used in conjunction with fractionation by HPLC

Non-Specific Analytical Techniques

Non specific tests such as TOC and NVR can give an indication of the total level of extractables in mass or concentration (Table 3).

Other Analytical Techniques

Headspace GC: The sample is heated in a closed system and volatile compounds in the sample are vaporized. Headspace-GC is able to detect residual solvents and volatile extractables.

Inductively Coupled Plasma – Mass Spectrometry (ICP-MS): Twenty or more metals can be analyzed. Metals are most often a concern when additives that contain metals such as fillers and colorants are used.

Identification of Extractables and Leachables

It is recommended to identify and quantitate detected extractables and leachables. LC-MS and GC-MS form the basis for identification because the retention times and mass fragments are usually correlated to an individual extractable/leachable. There are commercial databases to aid in identification with GC-MS. Individual laboratories will typically generate internal databases of commonly found extractables and leachables not found in the commercial databases.

TABLE 3 Non-Specific Techniques for Extractables and Leachables Analysis

Method	Description	Advantages	Limitations
NVR	The extraction solvent is evaporated and the residual mass is weighed. NVR is described in USP <661> where sample is evaporated at 105°C	• Gives total mass of non-volatile extractables • Relatively simple technique	• Any extractables that have significant vapor pressure at the boiling point of the solvent will be evaporated and therefore not measured accurately • Volatile compounds are ignored • Cannot be used with formulations that have significant non-volatile components such as buffers • Thermally unstable compounds may be degraded
TOC	Inorganic carbon is purged and then organic carbon is oxidized to form carbon dioxide which is measured	• Measures concentration of carbon based extractables	• Cannot be used with formulations or solvents with significant organic content
pH	Measurement of pH before and after extraction	• For non-buffered solutions, a change in pH can indicate an extractable	• Does not indicate reason for pH change

The mass fragments from LC-MS and LC-MS-MS are also used to identify leachables and extractables although there are no commercial databases available. A qualified laboratory, however, can interpret the spectra to facilitate identification. Identification of HPLC peaks can also be accomplished by isolating the peak, concentrating it and then analyzing by GC-MS and FTIR. There are commercial databases containing over 200,000 FTIR and GC-MS spectra.

Ideally, once an extractable/leachable is identified, the compound is procured and the identity confirmed by comparing the mass spectra and retention times using LC-MS or GC-MS. With this information, the confidence of the identity is absolute.

Finally, it is not always possible to positively identify some extractables and leachables. This can occur when the unknown extractable/leachable is an oligomer or series of oligomers for which the precise molecular weight cannot be determined. Or, it may be a degradation product of an additive for which there is not a database match and for which a standard cannot be purchased. In such cases, in accordance with ICH Q3A (R2) (2006), it is acceptable to show the efforts that have been made to identify the compound. When absolute identification is not possible, a general chemical classification can be made (e.g., siloxane, aliphatic acid).

Quantitation of Extractables and Leachables

If the identity of an extractable or leachable is confirmed by comparison to a procured reference standard, then the quantity in the filter extraction sample can easily be determined. A quantitation method should be validated according to the ICH Q2(R1) guidelines (ICH, 2005). Alternatively, the quantity can be estimated by bracketing multiple injections of the filter extract with multiple injections of the same solution spiked with the identified extractable at high and low concentration. Appropriate statistics can be used to show that the concentration of the compound in the filter extract is bracketed by the high and low concentration standards.

If the compound cannot be procured, the identified leachable can be quantitated using a compound of similar chemical structure and/or character that will produce a similar peak response in the specific analysis method. For instance, an extractable/leachable that is an oligomer, can be quantified with the base monomer. Or, a degradation product of a phenolic antioxidant might be quantified with the antioxidant itself. It is important to scientifically justify any chemical substitutions made for quantitation.

FTIR of NVR

The analysis of the NVR from a filter extraction using FTIR has been discussed in the literature (Weitzmann, 1997a,b; Stone, 1994) as a way to both quantitate and identify extractables/leachables. The user is cautioned about this method because of limits to the scientific relevance.

1. Only analyzing NVR eliminates all volatile and many or most semi-volatile extractables depending on the boiling point of the extraction solvent which can be as high as 187°C for propylene glycol.
2. FTIR can only distinguish the major non-volatile components from the remaining mixture and only with limited certainty.
3. Components present in lower concentrations may not be detected at all.

FTIR is a powerful technique when used to analyze pure samples as discussed earlier. However, it has clear limitations when analyzing mixtures.

Example of HPLC Analysis of a Leachable Test Sample

Figures 5A and 5B show the HPLC chromatograms of the blank control (pharmaceutical formulation without filter) and the filter extract. In Figure 5A, there is a large product peak between 1 and 3 minutes and a small product peak at 34 minutes. In the filter extract (Fig. 5B), the same two product peaks are detected. A unique peak at 18 minutes is also detected. The 18-min peak is related to a leachable from the filter.

It is possible for a filter leachable to elute within the first few minutes of the chromatographic analysis where the large product peak elutes. Since the chromatographic column used in this example does not retain hydrophilic compounds chemically, many product related peaks elute rapidly and within the column void volume. The void volume is defined as the volume of mobile phase required to carry an un-retained component

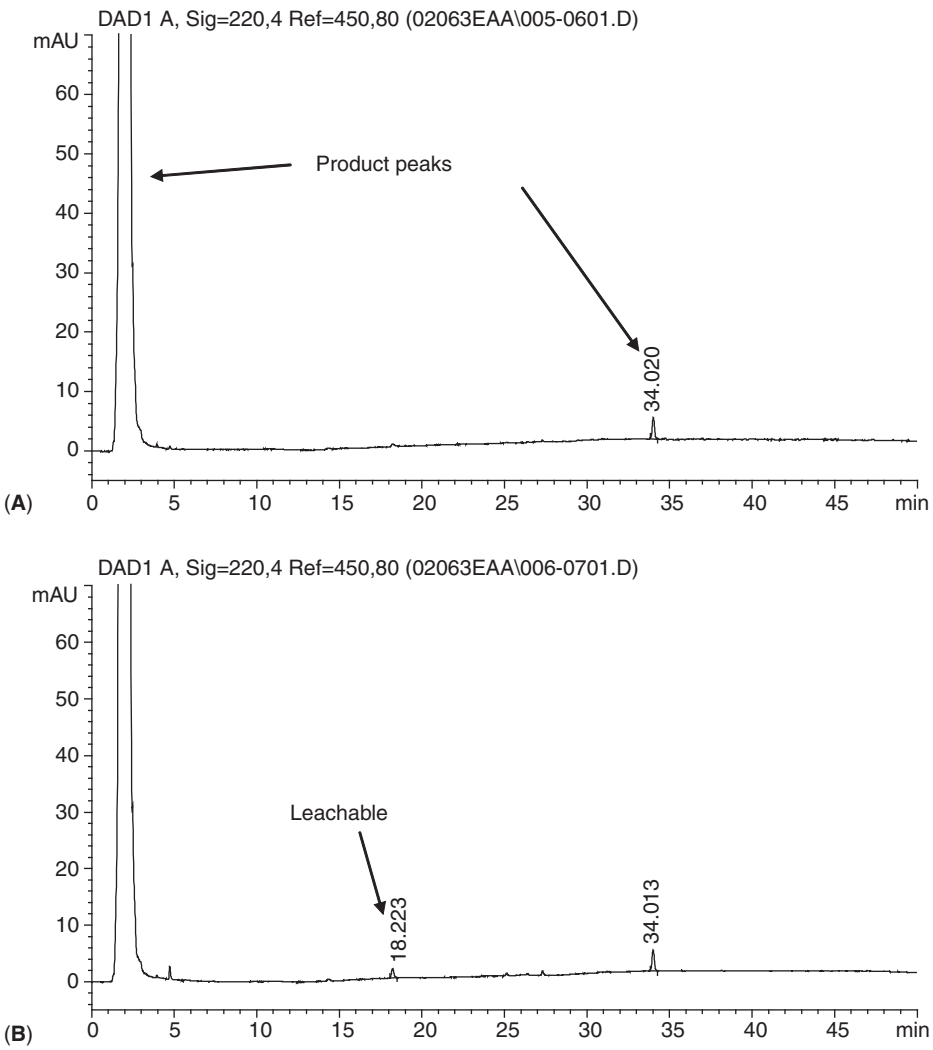


FIGURE 5 (**A**) HPLC with UV detection of a blank control from an actual formulation. (**B**) HPLC with UV detection of a filter extract from the same formulation.

through the HPLC system. Since most filter leachables are at least somewhat hydrophobic, it is not likely that a leachable will elute before or within the first product related peak. The second product related 34 minutes is sufficiently small whereby a co-eluting leachable would be detectable either due to a larger, broader peak or a shoulder on the peak.

Eliminating Analytical Interference in Leachables Testing

Most extractable solvents are chosen because they have minimal or at least predictable analytical interference. However, many pharmaceutical formulations used in leachables testing have compounds that can interfere with analytical techniques such as HPLC and GC-MS. Examples of compounds that cause analytical interference are proteins, surfactants, amino acids and molecules that contain unsaturated carbon bonds. It is recommended to analytically pre-screen suspect process streams in order to determine which may have irresolvable analytical interference. Once determined, alternative testing options for these specific process streams can be evaluated and determined. Since the alternatives may require more time to perform it is best to know this as early in the validation program as possible.

Technical Report 26 (PDA, 1998) also addresses the complications of detecting leachables in the presence of the components of the pharmaceutical formulation by stating:

> When the product formulation precludes the use of standard analytical methodology, a suitable model may be used to measure the levels of extractables.

It is possible to predict which process streams may produce analytical interference based on the chemical structure. Figure 6A shows the structure of Histidine, a chemical which produces significant UV interference when analyzed by HPLC-UV. Figure 6B shows the structure of Triton X-100, a surfactant which produces significant UV interference even at fairly low concentrations. In both examples, the UV interference is caused by the presence of the ring structures.

There are several options to resolve problems with analytical interference. First, one can design the analytical method to separate potentially interfering compounds. For example, a reversed-phase HPLC method can be designed to allow the hydrophilic formulation components to pass through the HPLC column unimpeded while the column retains the leachables that have a more organic nature. This is an acceptable direction because most filter leachables are not hydrophilic.

Second, by using more than one analytical technique it is more likely a leachable will be detected. By using complementary methods such as HPLC and GC-MS the interfering compounds in one instrument may not interfere the same way in the other instrument.

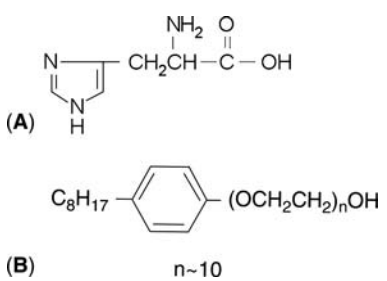


FIGURE 6 (**A**) Structure of histidine. (**B**) Structure of Triton X-100.

Third, the interfering compound may be removed using a sample preparation technique. Standard techniques to remove the interfering compounds are solid phase extraction (SPE), liquid–liquid extraction (LLE) and size exclusion chromatography.

When preparing a sample to remove analytical interference, the method used must be qualified each time it is used. This can be accomplished by spiking the pharmaceutical formulation with compounds that are known to extract from the material being tested. The method is qualified if all of the spiked compounds are sufficiently recovered after sample preparation.

Examples of Analytical Interference Removal

1. *Histidine*: Use of SPE demonstrates the removal of analytical interference from the histidine sample. SPE uses a column prepacked with silica beads that are treated with an organic layer with an 18 carbon chain length. After the column is conditioned with water and methanol, a sample of the pharmaceutical formulation is pulled through the column with mild vacuum. The leachables adhere to the hydrophobic column while the more hydrophilic product passes through the column unimpeded. The leachables are then eluted with a solvent such as methanol, acetonitrile or methylene chloride.

   Figures 7A and 7B show an example of an aqueous solution containing 2.5% Histidine. Figure 7A shows the direct injection analysis using RP-HPLC with UV-detection. In the first 12 minutes it is not possible to detect leachables because of the analytical interference. Figure 7B shows the formulation spiked with known potential leachables and the analytical interference removed using SPE.

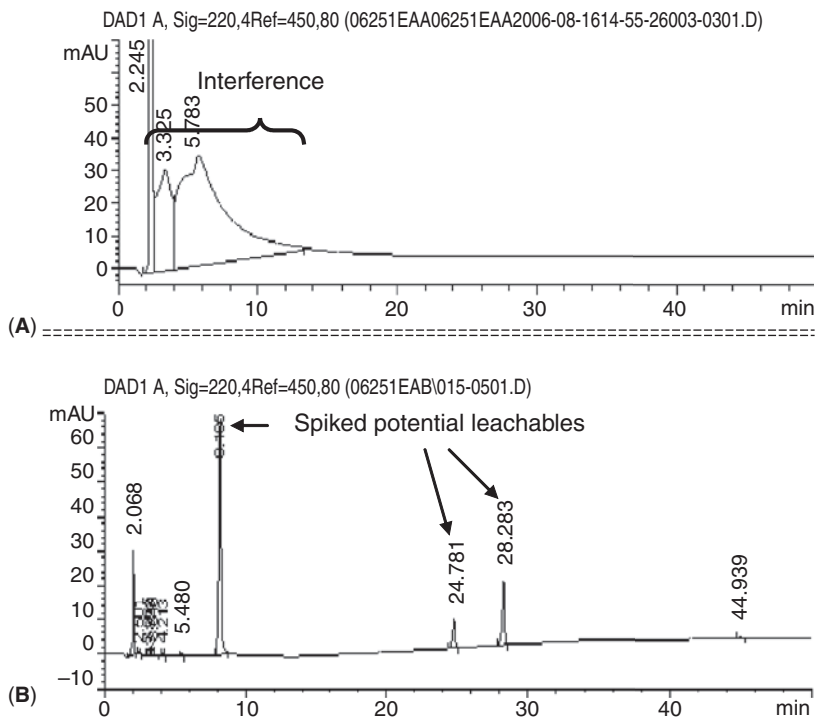


FIGURE 7 (**A**) HPLC-UV of histidine formulation. (**B**) HPLC-UV of histidine formulation with interference removed by SPE.

Alternatives to Standard Leachables Testing

There are situations when it is not possible to test with the actual formulation. In these cases, it is wise to use the best available science to choose an alternative test method.

Cases where testing with the formulation cannot be done include:

1. formulations which cause irresolvable analytical interference;
2. formulations that are cytotoxic;
3. formulations that are prohibitively expensive.

In these cases, it is often acceptable to modify the actual formulation slightly and allow for successful analysis. For example, removal of a cytotoxic compound that is present at a low concentration will likely have minimal effect on the potential of the formulation to leach organic compounds from the polymer. This is especially true if other ingredients in the formulation dominate the potential to extract leachables. The same approach may be used for formulations that are prohibitively expensive.

There are certain formulations that lead to irresolvable analytical interference or negatively alter the analytical performance of the instruments thereby making the results unreliable. An example of a common additive often used as a solubilizing agent in pharmaceutical formulations is Triton X-100. Triton X-100 (Fig. 6B) is a surfactant with an eight carbon alkyl chain (saturated hydrocarbon), a phenyl group (aromatic-ring), 10 unit polyoxyethylene chain (multiple ethyl esters) and a hydroxyl group. The combination of oliophilic (oil loving) groups and hydrophilic (water loving) groups make it ideal to keep drugs or biopharmaceutical ingredients that have limited water solubility in solution. The same properties that make Triton X-100 useful for solubilizing drugs also make it effective at solubilizing leachables from polymers. In addition, sample preparation steps such as SPE and LLE retain the Triton X-100 along with the leachables.

Figure 8A shows an HPLC chromatogram of a 20% aqueous solution of Triton X-100. The analytical interference is dramatic and in this specific example, ruined the HPLC column and contaminated the entire HPLC system. Figure 8B shows the HPLC chromatogram for 0.1% (1000 ppm) Triton X-100. There is substantial interference from 13 to 17 min. There is still significant interference at 0.01% (100 ppm) as shown in Figure 8C. Only when the concentration is reduced to 0.001% (10 ppm) is the analytical interference reduced to negligible as shown in Figure 8D.

When there is irresolvable analytical interference there are several approaches to meeting the leachables testing requirement. One approach is to substitute or mimic the interfering compound. A second approach is to use data from extractables modeling studies of the filter and estimate the correlation to the actual formulation.

Example of the Mimic/Substitute Approach:

1. If the interference is restricted to a narrow range as in the case demonstrated by 0.1% Triton X-100 (Fig. 8B), the leachable test with the actual formulation can still be performed knowing that a narrow range (4 minutes out of 50 minutes) cannot be used to detect leachables. The remaining 46 minutes are unobstructed and therefore useful.
2. To address the four minutes of interference, a second extraction would be performed with the Triton X-100 substituted. The Triton X-100 concentration would be removed or lowered and another compound substituted as a mimic that has similar properties. In this case, ethanol or IPA would be substituted. To make it worst-case, the concentration of the mimic can be higher than the compound it is replacing. To mimic 0.1% Triton X-100, one might use 1% or 10% ethanol.

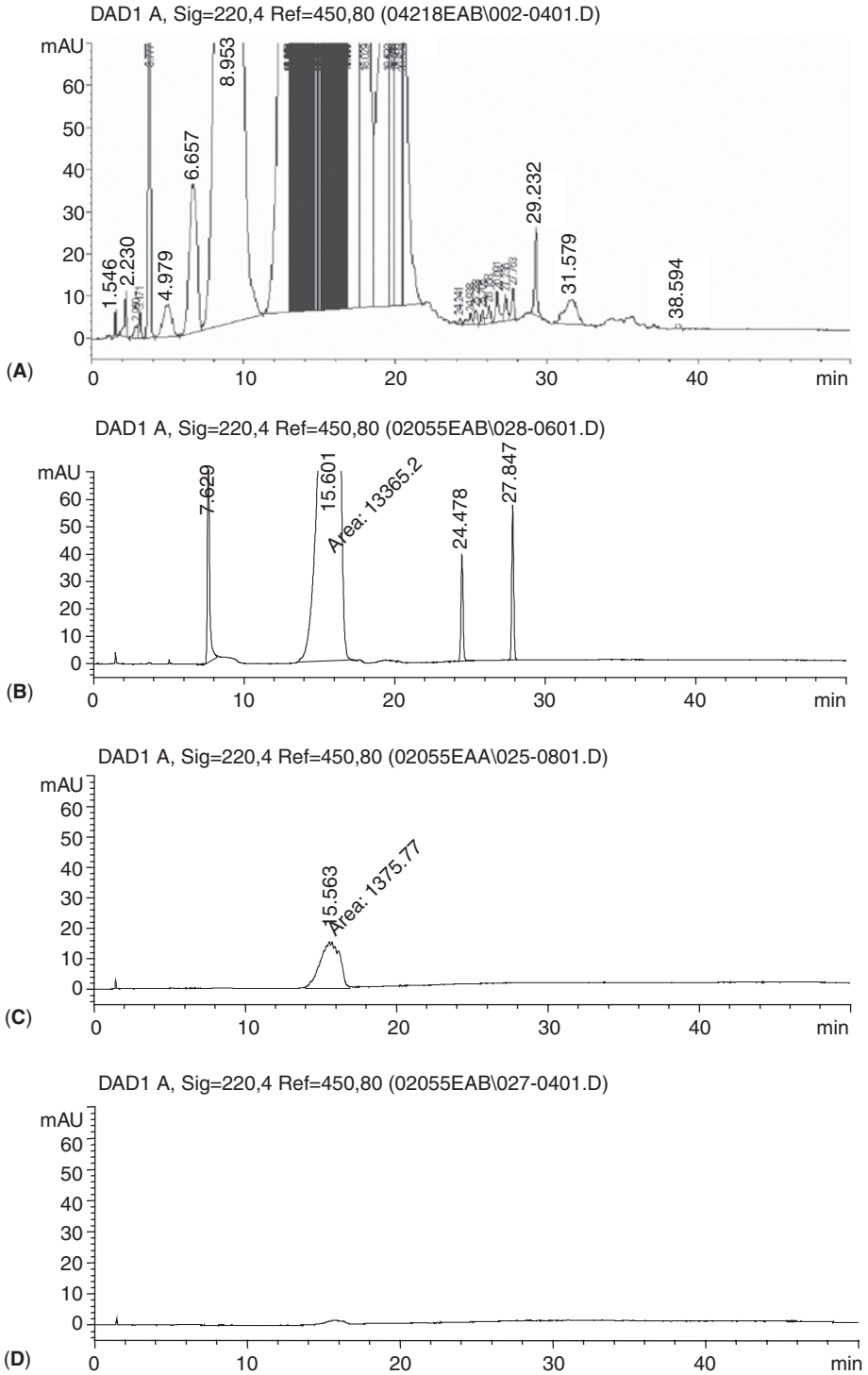


FIGURE 8 (**A**) HPLC-UV of 20% Triton X-100. (**B**) HPLC-UV of 0.1% (1000 ppm) Triton X-100. (**C**) HPLC-UV of 0.01% (100 ppm) Triton X-100. (**D**) HPLC-UV of 0.001% (10 ppm) Triton X-100.

Modeling Leachables

A second approach to address leachables when the actual formulation precludes standard analytical techniques is to use existing extraction data that is provided by the filter manufacturers. In lieu of testing with actual product, some filter manufacturers have data from a variety of solvents that are performed at conditions more typically found in a process instead of the more extreme conditions that are used for standard extractables testing. This method is discussed in detail by others (Weitzmann, 1997a,b; Stone, 1994). Modeling follows these steps:

1. identify the significant constituents to be modeled;
2. examine the functional groups in each constituent;
3. assign each constituent or functional group within a constituent to a solvent group;
4. apply data from the representative solvents to the worst case for each group.

Typical solvents and functional groups are suggested to be (Weitzmann, 1997a):

1. water (for groups such as cresols, fluoroalkanols);
2. ethanol (for aliphatic alcohols);
3. dimethylforamide (for amide, glycol ethers);
4. propylene glycol (for glycols);
5. methylene chloride (for methylene chloride and ethylene chloride);
6. 2-ethoxyethanol (for polyethers and dioxane);
7. acetone (for aliphatic esters and ketones).

One advantage of modeling is that the performance of leachables testing is usually not required because it relies on a database of extractions. Another consideration is there is historic record to modeling being accepted by the regulatory bodies.

Disadvantages of modeling include:

1. There is no scientific correlation between the model solvents and the actual drug formulation.
2. Use of solvents such as 100% ethanol, dimethyl foramide and methylene chloride can lead to over prediction of the leachables.
3. The tested filter may not be the same configuration as the actual process filter.
4. Modeling is often used as a substitute to actual product testing even when standard analytical methods do not preclude the use of the actual product.

EXTRACTABLES AND LEACHABLES SUMMARY

In summary, start early in the product lifecycle to develop the filter validation program which includes extractables/leachables testing. Consider the following steps to create a successful program:

1. include critical stakeholders in the planning and implementation process;
2. perform a filter risk assessment;
3. obtain extractable data from manufacturers;
4. obtain verification of materials of construction from filter manufacturers;
5. determine which filters require validation;
6. do a grouping analysis to possibly reduce the number of tests;
7. understand the chemical nature of the process streams being validated;

8. consider pre-screening process streams to find irresolvable analytical interferences so alternative testing can be decided and addressed early;
9. perform extractable and/or leachable testing using suitable analytical techniques;
10. identify and quantitate detected leachables.

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15

Endotoxin, Limulus Amebocyte Lysate, and Filter Applications

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INTRODUCTION

The advent of injection therapy signaled a dramatic change in the way we think about pharmaceutical production and its associated quality control. Before injection therapy, all medications were administered either orally or topically, but now physicians of the day could administer their preparations directly into places they had never been before.

Anatomically, the route oral medications take once ingested, through the alimentary canal, is outside our body. Developmentally, this design template for the organization of living systems has had hundreds of millions of years to evolve both physical and biochemical defenses to protect us from the majority of the poisons we ingest. Similarly, our intact skin acts as a safety barrier for topical medications. Injection therapy bypassed all these defenses.

Early experimentation in the mid 1700s and 1800s gave rise to the notion that the injection of fluids was always associated with the onset of fever (von Haller, 1757; Gaspard, 1822). While the nature of the fever inducing substance remained unknown, Billroth (1862) applied the term *pyrogen* to any material which when injected induces fever. *Pyrogen* describes an *in-vivo* characteristic and a solution that contains a fever-inducing substance is said to be pyrogenic. Panum (1874) postulated the existence of a heat-stable pyrogenic material present in the injected material as the causative agent.

Around the same time, the work of Joseph Lister (1827–1912) and Louis Pasteur (1822–1895) were teaching us we are not alone; we share our world with the world of the microbe. Subsequent investigations on the nature of the fever-inducing material suggested that it was indeed of bacterial origin (Billroth, 1962; Burdon-Sanderson, 1876; Jona, 1916; Centanni, 1921) and exclusively associated with Gram-negative bacteria (Westphal et al., 1977). The word *endotoxin* was reportedly proposed by Pfeiffer to describe the membrane-associated toxin of *Cholera* (Pearson, 1985). Gram-negative bacteria thrive in aqueous environments. Since water and aqueous solutions are use universally in pharmaceutical manufacturing, it was thus logical to conclude that endotoxin associated with Gram-negative bacteria was the pyrogenic material found in injectables.

By definition the word *endotoxin* represents the pyrogenic material associated with Gram-negative bacterial. Gram-negative bacteria contain a second outer membrane, not present in Gram-positive bacteria, which serves to prevent the internalization of the

Gram stain. Gram-negative bacteria are Gram-negative, not on the basis of what they are missing or lacking, but by what they contain that Gram-positive species do not. Endotoxins, in their natural state, are fragments of the outer membrane of Gram-negative bacterial cell wall, literally lumps and chunks of Gram-negative bacteria. Both viable and non-viable Gram-negative bacteria contain active endotoxin. Sterile does not mean endotoxin-free.

The studies of Wechselmann (1911), Muller (1911), Samelson (1913), and Bendix and Bergmann (1913) raised continued concern over the presence of endotoxin in pharmaceuticals. Hort and Penfold (1912a,b,c) expanded the findings that a Gram-negative bacterial substance was responsible pyrogenicity. These workers are also credited with designing the first rabbit pyrogen assay (Pearson, 1985).

Subsequently, confirmatory observations by Seibert (1923, 1925), Seibert and Mendel (1923), and (Rademaker (1930, 1933) reinforced the concept that bacterial contamination was present in all pyrogenic injectables, and that through the use of careful techniques to avoid bacterial contamination, non-pyrogenic injectables could be produced. Their work lead to a collaborative study, which standardized the rabbit pyrogen test, and allowed pyrogen testing to attain pharmacopeial recognition in 1942, and in doing so, the testing for the presence of endotoxin became an integral part of pharmaceutical manufacturing and quality control.

The rabbit pyrogen test remained the exclusive official pyrogen assay referenced in the U.S. Pharmacopeia (USP) for over 25 years. However, events culminating in the late 1950s and early 1960s were to lead to a significant alternative to the rabbit pyrogen test for determining the pyrogenicity of pharmaceuticals and medical devices. That alternative was developed using the blood of the American horseshoe crab *Limulus polyphemus*.

ENDOTOXIN AND LPS

Chemically, the toxic molecule found in the outer membrane of Gram-negative bacteria is lipopolysaccharide (LPS). It is a linear molecule containing 4–7 even numbered carbon fatty acids chains linked to a long chain of repeating sugars. The composition of the polysaccharide chain differs from specie to specie. The fatty acid potion of the LPS molecule, referred to as Lipid A, is relatively conserved. It is the Lipid A potion of the LPS molecule which is pyrogenic. The most common method to isolate LPS from Gram-negative bacteria cell wall is hot phenol extraction.

In the presence of the divalent cations calcium and magnesium endotoxin molecules aggregate to form vesicles and bilayers ranging from 3×10^5 to 1×10^6 Da. Chelation of divalent cations reduces the size of endotoxin aggregates to 2×10^4 Da (Sweadner et al., 1977).

However, from a practical point, endotoxin can be considered to have a molecular weight 10^6 Da in aqueous environments. This is the approximate molecular aggregate most commonly encountered in large-volume parenterals and medical device rinse solutions (Williams, 2001).

LIMULUS AMEBOCYTE LYSATE

Biochemistry

The biochemical basis for the use of the blood of the horseshoe crab, *Limulus,* for the detection of endotoxin is based entirely on the coagulation reaction inherent in *Limulus*

blood. Howell (1885), Loeb (1902, 1909), and Blanchard (1922) initially reported the ability of *Limulus* blood to form a gelatinous clot as early as 1885. These early investigators showed that coagulation was induced by foreign substances and that the circulating amebocyte was involved in the reaction (Loeb, 1928). The association between endotoxin and coagulation was first reported in 1956 when Bang (1956), investigating bacterial defense mechanisms in marine invertebrates, reported that infecting the horseshoe crab with *Vibrio,* a Gram-negative bacteria, caused a fatal intravascular coagulation. By 1964, Levin and Bang (1964) had demonstrated that extracts of the circulating amebocytes would gel on the presence of Gram-negative bacterial endotoxin. These extracts became known as *Limulus Amebocyte Lysate* (LAL).

Limulus blood has always held a certain fascination because of its bluish appearance. The blue color is due to hemocyanin, a copper-based oxygen acceptor. The hemocyanin is extra-corporeal, meaning not contained in any blood cell, and remains in the plasma after centrifugation of whole blood. The amebocyte is the only cell present in *Limulus* blood. The amebocyte contains all the components of the entire coagulation system (Leob, 1902; Levin and Bang, 1964; Dumont et al., 1966). Cell-free hemolymph does not gel in the presence of endotoxin nor does it enhance the endotoxin-mediated reaction associated with amebocyte extracts (Levin and Bang, 1964, 1968). Lysates from washed amebocytes can be prepared by lysis in hypotonic solutions (Levin and Bang, 1968; Jorgenson and Smith, 1973), by physical disruption upon mechanical shear (Takagi et al., 1979; Yin et al., 1972), or by freeze-thaw cycles (Levin and Bang, 1968; Takagi et al., 1979). The sensitivity of amebocyte lysates to endotoxin can be increased by subsequent extraction with organic solvents (Sullivan and Watson, 1974) or treatment with zwitterions.

Young et al. (1972) presented a simplified description of the Limulus coagulation reaction sequence. They fractionated amebocyte lysate into two major fractions using gel filtration chromatography. Fraction I, the column void, contained a heat-labile component representing material >75kD. Fraction II represented a heat-stable component of lower molecular weight. Neither fraction alone showed any reaction to endotoxin. However, a mixture of both fractions regenerated the reactivity to endotoxin seen in unfractionated lysate. Incubation of Fraction I material with endotoxin prior to the addition of material from Fraction II greatly accelerated the time course of coagulation. This supported the two-step mechanism originally proposed by Levin and Bang (1968). A proenzyme, the Fraction I material, is activated by the presence of endotoxin. This active enzyme subsequently catalyzed the conversion of a clotting protein (Fraction II material) into an insoluble gel. The rate-limiting reaction is the activation of the proenzyme by endotoxin, with the concentration of active enzyme being proportional to the initial concentration of endotoxin.

The coagulation reaction is not as simple as the two-step reaction originally proposed. It exists as a multi-component activation cascade, which is initiated by endotoxin and terminates with gelation. While the majority of the biochemical analysis has been performed by Japanese workers using lysate from another species of horseshoe crab, *Tachypleus tridentatus* (Nakamura et al., 1983; Morita et al., 1985), their observations are thought to apply to *Limulus* as well. The first coagulation factor, Factor C, directly interacts with endotoxin to form activated Factor C, represented as *Factor* $\overline{C}$. An anti-LPS factor, found both in *Limulus* and *Tachypleus* inhibits the interaction of Factor C by endotoxin (Sullivan and Watson, 1974). Factor $\overline{C}$ possesses enzymatic activity, which activates Factor B. Factor $\overline{B}$, the active form of Factor B, is the responsible for activating the proclotting enzyme.

The clotting protein, coagulogen, is the last component in the coagulation cascade. It comprises almost half of the total protein present in amebocyte lysate (Young et al., 1972). The active clotting enzyme, a serine protease (Tai and Kul, 1977), hydrolyzes specific peptide bonds within the coagulogen to form a shorter peptide, coagulin (Tai and Kul, 1977; Nakamura et al., 1976). Once generated, the coagulin self-associates forming a three-dimensional lattice structure and eventually gels.

The catalytic nature of each activated component in the coagulation cascade serves to amplify the next step in turn. This amplification most probably results in the extreme sensitivity of LAL to endotoxin. All commercial lysates are capable of detecting picogram (pg) quantities (10^{-12}grams) of endotoxin.

In addition to the endotoxin mediated cascade, which activates the proclotting enzyme, another protein component, Factor G, represents an alternative activation pathway (Morita et al., 1985). Factor G is present in both *Tachypleus* and *Limulus* (Morita et al., 1985) and is activated by β1-3 and β1-4 d- glucans. Glucans are polymers of glucose, found in the outer membranes of algae, fungi and as a breakdown product of cellulose. Factor G directly activates the proclotting enzyme, which in turn causes the gelation of coagulogen. The presence of glucans in samples tested for endotoxin using LAL causes false positive results. The use of cellulosic filters in pharmaceutical manufacturing has been shown to result in false positive LAL results. All commercial LAL manufactures supply reagents which either block the glucan reaction or, based on manufacturing, are not sensitive to glucans.

Methodology

Gel-Clot Assays

The most commonly employed LAL method is the gel-clot assay. This assay utilizes the entire endotoxin-mediated cascade in addition to the clotting protein to produce a gelatinous clot after incubation with endotoxin. Basically, equal volumes of sample and LAL (typically 0.1 ml each) are combined in a 10×75 mm glass tube. After an incubation period of 60 min at 37°C, the tubes are inverted 180°. A positive result in indicated by a clot, which withstands the inversion. By titrating the lysate with an endotoxin of known potency, the minimum concentration required to yield a positive clot can be determined. The minimum endotoxin concentration, or end point, is referred to as the lysate sensitivity.

The gel-clot assay can be used as a purely qualitative limits test to rank samples as either positive or negative, that is, greater than or less than the lysate sensitivity. However, by titrating positive samples one can obtain a semi-quantitative measure of the endotoxin concentration in unknowns by multiplying the last positive sample dilution by the lysate sensitivity.

The gel-clot assays suffers from the disadvantage that they are at best semi-quantitative and require preparing multiple dilutions of samples to determine a positive/negative end point. Various alternative methods have been developed to yield more quantitative results.

Turbidimetric End Point and Kinetic Assays

During the process of clot formation, the reaction mixture becomes increasingly more turbid as it contains a larger portion of insoluble clotting protein. The turbidimetric assays measure this increase in turbidity as a function of endotoxin concentration. Endotoxin concentrations in unknowns are determined by comparing the resultant turbidity to

a standard curve. Since turbidity is a precursor to gelation, these assays are more sensitive to endotoxin concentration. Turbidimetric assays have been described which measure turbidity at the end of a fixed incubation period, the turbidimetric end point assays, or measure turbidity continually during the reaction utilizing reaction rates or onset time to a fixed turbidity, the turbidimetric kinetic assays. The latter has the advantage that it can quantitate endotoxin over a greater concentration range.

Chromogenic End Point and Kinetic Assays

All the methods previously described utilize the natural clotting protein as the substrate for the endotoxin-mediated coagulation cascade, and employ various methods to detect the gelation reaction. The chromogenic assays still utilize the initial portions of the enzyme cascade, but substitute a synthetic chromogenic peptide as substrate for the activated clotting enzyme in place of the clotting protein (Friberger et al., 1982; Scully et al., 1980). The chromogenic substrate is hydrolyzed by the clotting enzyme releasing the terminal chromogenic moiety and generating a yellow color.

The chromogenic end point assay is normally performed in a two-step assay, where LAL and sample are incubated, followed by the addition of substrate. The resultant absorbance is determined spectrophotometrically after the reaction is stopped with acetic acid. Like the other quantitative procedures, endotoxin in unknowns is determined from a standard curve. The assays are rapid, less than 20 min (Friberger et al., 1982), and extending the initial incubation of LAL with sample can increase the sensitivity to endotoxin.

The chromogenic kinetic assay utilizes a single co-lyophilized LAL/substrate reagent, which is incubated with the sample and monitored spectrophotometrically for the appearance of hydrolyzed substrate. Endotoxin in unknowns is interpolated from standard curves, which are linear over a five-log concentration range.

Recombinant LAL Assays

The first component of the endotoxin-mediated cascade, Factor C, has been successfully produced recombinantly (Ding et al., 1995). This recombinant Factor C is activated by endotoxin and can be detected using a fluorogenic substrate. An end point recombinant Factor C assay is commercially available with a detection limit of 0.01 EU. Since Factor C is not activated by glucans, the recombinant LAL assay is endotoxin-specific.

REGULATORY ASPECTS

The development of regulatory policies for LAL use has been a collaborative effort between national and international regulatory agencies, including the U.S. Food and Drug Administration (FDA), the U.S. Pharmacopeia Committee of Revision (USP), the European Pharmacopoeia Commission (EP), the Society of Japanese Pharmacopoeia (JP), lysate manufacturers, and the parenteral drug and medical device industry. Throughout development of the methodology, regulatory agencies have taken their lead from data presented by LAL researchers, issuing or modifying documents in response to scientific advances made by industry.

The first regulatory acknowledgement of LAL as a useful tool for the detection of endotoxin came in 1973, when FDA announced that LAL reagent was to be considered a biological product, subject to license (FDA, 1973a,b). The next regulatory notice

appeared in the Federal Register on November 4, 1977, by which time the pharmaceutical industry has gained considerable experience with the test (FDA, 1977). The largest body of data initially came from those involved in testing large volume parenterals and medical devices (Jorgensen and Smith, 1973; Weary and Baker, 1977), but data from other segments of the parenteral industry, including antibiotics (McCullough and Scolnick, 1976; Newsome, 1977), vaccines (Rastogi et al., 1977), antitumor agents (Siegel et al., 1976), and other small volume parenterals (Sullivan and Watson, 1975; Murata et al., 1976) began to appear in the literature. In the November 4 notice, FDA outlined conditional approval for the use of LAL as a final release test for licensed biological products and medical devices.

Before the in vitro LAL assay could fully replace the in vivo Rabbit Pyrogen assay, an estimate of the average pyrogenic dose in rabbits needed to be established (Cooper et al., 1971; Eibert, 1972; van Noordwijk and DeJong, 1976; Tomasulo et al., 1977). The HIMA study, as reported by Dabbah and collaborators (Dabbah et al., 1980), suggested the average pyrogenic dose in rabbits was 1 ng of *Escherichia coli* O55:B5 endotoxin per kilogram total body weight. Based on this study, the maximum endotoxin exposure was established for humans as 1 ng/kg body weight.

Initially, the potency of endotoxin standards was stated by weight. The first widely distributed FDA endotoxin standard, EC-2, was supplied in 1 μg aliquots (Rudback et al., 1976). With subsequent productions of replacement endotoxin standards it was realized that the activity of endotoxin on a weight basis was not consistent. In 1983, a FDA sponsored collaborative study defined and established the endotoxin unit, or EU (Hochstein et al., 1983). The endotoxin unit is defined as the activity of 0.2 ng of EC-2, or 5 EU/ng. One vial of EC-2 (1 ug) therefore contained 5000 EU. The 1 ng/kg maximum endotoxin exposure limit for humans became 5 EU / kg. The current Reference Standard Endotoxin, EC-6 (USP lot G) also contains 1 μg of endotoxin but is labeled as 10,000 EU (Poole and Mussett, 1989). EC-6, as supplied, is 10 EU/ng. It is important to note that the activity of endotoxin per ng varies depending on the source of the endotoxin and its purification.

In response to continued research and data acquisition by the parenteral industry, the U.S. Pharmacopeia published its first chapter on the bacterial endotoxins test in USP XX. The focus of this chapter was on the development of a single, consistent gel-clot method, including suggested procedures for reagent label claim verification, product validation, and routine testing. The USP chapter did not, however, suggest endotoxin limits for the release testing of compendial articles, and did not offer the LAL test as a replacement for the USP rabbit pyrogen test.

In 1987, FDA published its "Guideline on the validation of the LAL Test as an end-point release pyrogen test for human and animal parenteral drugs, biological products and medical devices" essentially allowing the LAL test to be used in place of the Rabbit Pyrogen test in final release testing of parenterals (FDA, 1987). In 1990, the USP began substituting endotoxin limits tested using LAL in place of the Rabbit Pyrogen Test requirements in parenteral drug monographs.

To date, the Bacterial Endotoxin Test chapter in all the major pharmacopeias worldwide has been harmonized and references all the qualitative and quantitative LAL methods.

DEPYROGENATION AND ENDOTOXIN REMOVAL

Depyrogenation may be defined as the elimination of all substances capable of producing a fever. The pyrogenic substance of most interest to the biopharmaceutical industry is

endotoxin (with LPS as the active component). Endotoxins are heat-stable and are not destroyed by steam sterilization (autoclaving). Elimination of endotoxin is accomplished either by inactivation or physical removal.

The USP chapter "Sterilization and Sterility Assurance of Compendial Articles" recognizes the value of validated processes designed to eliminate endotoxin. The USP chapter states that an acceptable depyrogenation process should be able demonstrate at least a three-log (10^3) difference between the recoverable input endotoxin ≥1000 EU and any residual endotoxin present after processing.

The choice of a depyrogenation process is dictated by the nature of the material being processed. Depyrogenation by inactivation of the toxic lipid A portion of the LPS molecule can be accomplished by a number of methods, including long exposure to high temperature, acid/base hydrolysis, oxidation, alkylation, moist heat, and ionizing radiation (Weary and Pearson, 1988; Robertson et al., 1978; Tsuji and Harrison, 1978; Akers et al., 1982; Ludwig and Avis, 1990). These methods are perhaps best suited to those materials which are resistant to heat and chemicals, such as glass packaging components and metal processing equipment and utensils, they are most likely not suited to biopharmaceuticals.

Methods of depyrogenation by endotoxin removal are based on the physical characteristics of the endotoxin molecule in aqueous solution. These methods include ultrafiltration, affinity chromatography, dilution, absorption to a specific medium (such as asbestos, charcoal or charged filter media), hydrophobic attraction or reverse osmosis. These methods are widely used in production situations, where the product may be heat or chemically labile.

Although filters have been used to depyrogenate solutions of low molecular weight solutes, the true question is whether endotoxin can be separated from the molecule is interest. Sweadner et al. (1977) demonstrated that filtration could be used to depyrogenate, that is, reduce the endotoxin content by 3 logs. They reported that the LAL activity of a 1 ng/ml solution of *E. coli* LPS in water could be reduced greater than 4 logs by filtering through a 0.025μm pore size filter, suggesting that the endotoxin complexes in water are large enough to be retained. Large endotoxin aggregates require divalent cations. Addition of 5mM EDTA reduced the size of the endotoxin aggregates such that the 0.025 μm was no longer sufficient. A 10^6 molecular weight exclusion filter was required to reduce the LAL activity of a 1 ng/ml LPS by 4 logs. LPS reduced to its smallest subunit by surface active agents such as sodium deoxycholate, required a 10^4 molecular weight exclusion filter for similar endotoxin removal. The chemical composition of the solution, its effect of the aggregate size of endotoxin complexes, and the molecular weight of the target molecule all impact the choice of an appropriate filter matrix. If the target molecule of interest is stable in water and smaller than the 0.025μm, a 0.025-μm filter could be used to depyrogenate. Otherwise, filters with smaller pore sizes must be employed accompanied by the risk of also retaining the molecule of interest.

Charge modified filters have also been used to depyrogenate solutions. At pH > 2.0 endotoxins are negatively charged, and as such will attach to a positively charged matrix. Gerba and Hou (1985) evaluated the effects of positively filters on endotoxin removal. They demonstrated a greater than 3 log endotoxin reduction by filtering solutions as complex as newborn calf serum in 0.9% NaCl through a depth (cellulose-diatomaceous earth) filter. As expected, the extent of endotoxin removal was influenced by pH, as some breakdown in endotoxin retention was observed at pH 8.5 and above.

The filter company, Sartorius, is marketing a line of positively charged large pore size membrane filters under the name Sartobind™. Their application data indicates a

greater than three log reduction in endotoxin in solutions of natural gamma globulin and monoclonal antibody preparations by filtration.

No matter what the method of depyrogenation, validation studies can be conducted by adding a known amount of endotoxin to the material before processing and performing LAL assays on the material after processing to determine the efficiency of the procedure.

SUMMARY

Throughout the development of intravenous pharmaceuticals and implantable medical devices, pyrogenic reactions have always been closely associated with the introduction of foreign material into the body. The principal pyrogen in aqueous solution, one of the biggest concerns of pharmaceutical and medical device manufacturers, is a component of the cell wall of Gram-negative bacteria, called endotoxin. The observation that endotoxin caused gelation in extracts of *Limulus* amebocytes eventually was expanded to the development of an *in vitro* assay for endotoxin.

LAL contains a multicomponent enzyme cascade, which is initiated by endotoxin and terminates in coagulation. A variety of methods have been developed in an attempt to make the assay of endotoxin more simple, faster, more sensitive, or quantitative. Four methods are currently commercially available, namely the gel-clot, turbidimetric kinetic, the chromogenic end point, and the chromogenic kinetic assays.

LAL is recognized worldwide as an alternative to the *in vivo* rabbit pyrogen assay. The U.S. Pharmacopeia, FDA, European Pharmacopoeia, and Japanese Pharmacopoeia have guidelines for the validation and use of LAL in pharmaceutical and medical device manufacturing. These agencies have established limits for the amount of endotoxin allowable in products and endotoxin standards for use in the standardization of reagents.

Endotoxin removal, depyrogenation, can be accomplished by inactivation or removal. Endotoxin inactivation requires harsh chemical or environmental treatments. These methods are not suited for depyrogenation of biopharmaceuticals. Endotoxin removal can be accomplished based on size exclusion or charge affinity. Depyrogenation of large molecular weight material has been accomplished through the selection of the appropriate endotoxin removal process.

The use of LAL has proved invaluable in controlling the level of endotoxin in finished product. The endotoxin contribution of raw materials and packaging material can be monitored. In-process testing at critical production steps can identify additional sources of endotoxin contamination, and depyrogenation processes can be validated by assessing the degradation of endotoxin challenges. The speed, economics, and sensitivity of the LAL assays allow such an in-depth approach to the control of endotoxin in pharmaceuticals and medical devices.

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Limulus Amebocyte Lysate Assays and Filter Applications

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INTRODUCTION

Lipopolysaccharide (LPS), the biochemically purified endotoxic or pyrogenic structural molecule unique to the gram-negative bacterial cell wall, is easily detected and quantified using an in vitro assay referred to as the limulus amebocyte lysate or LAL test. LAL is a very sensitive reagent made from the soluble protein extract or lysate of horseshoe crab (genus *Limulus*) blood cells (amebocytes).

Endotoxins from gram-negative bacteria (classified on the basis of their cell wall structure) are the most common contaminants of water systems and biologically derived materials and are resistant to most methods of sterilization. Very low concentrations of endotoxins or LPS are toxic in vivo in blood or cerebral spinal fluid (FDA, 1987) and may affect the growth and function of vertebrate cells in vitro (Gould, 1984). Because LPS is a potential toxic contaminant of Pharmaceuticals, its detection in finished products and its control or removal from parenteral and medical devices is especially important to the pharmaceutical industry.

Applications for the LAL test also extend beyond those of the pharmaceutical industry because LAL detects LPS in a variety of backgrounds. LPS is detected when it is part of the bacterial cell or when it is "free" in solution as the result of bacterial growth, bacterial cell lysis, or chemical extraction. This means that the LAL test can be used to indicate both present and past contamination by the ubiquitous gram-negative bacteria, a group of organisms known for their ability to grow in the harshest environments.

Numerous papers address the structure, function, and bioactivity of lipopolysaccharides and the development and various applications of the LAL test. References are included here to provide initial sources of more information or as they pertain to specific topics, but the list is not meant to be exhaustive. The reader is encouraged to review these topics in more depth, especially if the LAL test is to be applied under conditions that have not been defined previously.

LIPOPOLYSACCHARIDES

The lipopolysaccharide molecule is an amphiphile. It has a hydrophilic polysaccharide attached to a hydrophobic lipid moiety. Molecules vary in molecular weight from a few

thousand to roughly 25,000 but are rarely found as unimolecular entities. Because of their amphiphilic nature, LPS molecules spontaneously aggregate in water to form membranes and vesicles. These particles reach molecular weights in the millions (Gould et al., 1991).

Lipid A is the smallest portion of the molecule that is endotoxic and reacts with LAL (Kotani and Takada, 1990). It has a hydrophilic backbone composed of two sugars and a hydrophobic region composed of nonhydroxylated and hydroxylated fatty acids (Rietschel et al., 1992; Takayama and Qureshi, 1992).

Lipid A has a negative charge that is neutralized in the cell membrane by cations, polyamines, and proteins with positive charges. In order to achieve stable and highly aggregated states, the charge on the LPS has to be neutralized. Purified endotoxins retain a mixture of cations that depends on the composition of the bacterial growth medium and on the method of purification (Coughlin et al., 1983b). The resultant bioactivity of the various salt forms of purified LPS or lipid A and their physicochemical behavior has been elucidated (Coughlin et al., 1983a; Galanos et al., 1979a).

Aggregate size and stability are also a function of the polysaccharide chain length (Lüdeiitz et al., 1986; Peterson and McGroarty, 1986). The differences in molecular weight among the LPS molecules even in a single bacterial cell are attributed to the heterogeneity of polysaccharide chain lengths (Hitchcock and Brown, 1983). Some types of bacteria make only short chain oligosaccharides as opposed to polysaccharides. This purified endotoxin is called a lipooligosaccharide and is extremely reactive on a weight basis (Rietschel et al., 1992).

Because of the nature of the LPS and the factors that influence its activity, chemical manipulation of solutions or surfaces contaminated with endotoxins may alter its structure and therefore its biological activity, including its reactivity with LAL. For example, mild acid hydrolysis (0.02 N sodium acetate buffer, pH 4.5, for 60 min at 100˚C) will yield a precipitable lipid A, free of the polysaccharide (core and O-antigen) but still toxic in a solubilized form (Rietschel et al., 1992). Acid hydrolysis of LPS with 0.1 N HC1 for 15–30 min at 100˚C yields a form of LPS that lacks the 1-position phosphate (Rietschel et al., 1992) and is no longer pyrogenic but retains its ability to cause tumor regression (monophosphoryl lipid A). Treatment with 0.2 N NaOH for 1 h at 100˚C liberates ester-bound fatty acids from lipid A, which alters the lipid A sufficiently to achieve true chemical depyrogenation. Treatment with highly oxidizing agents will also liberate fatty acids (Gould and Novitsky, 1985). Other treatments with acids, bases, salt solutions, or oxidizing agents, especially at temperatures less than 100˚C, may alter the structure of endotoxins in ways that are not clearly defined, but an altered structure invariably means altered activity.

The heterogeneity of endotoxins affects the strategies applied to their removal from surfaces and solutions. All endotoxins have some nonspecific affinity for surfaces via hydrophobic or ionic interactions. Depending on the strengths of those interactions, only those molecules or aggregates with greater affinity for the solute are going to rinse off a surface into that solute, and the rest will remain on that surface. Of those remaining after a water rinse, another fraction may rinse off in a saline solution, and another fraction only in solutions with a high or low pH. Surfactants and compounds with lipid-solubilizing capacities such as albumin will often solubilize endotoxins that will not be released with other compounds (Twohy and Duran, 1986). I have not been able to show convincingly that any one solution is capable of removing all surface-bound endotoxins, but once endotoxin has been removed with any one solution chemistry, subsequent further rinsing with the same solution does not release more (Gould, unpublished data). Therefore, prerinsing the purification or separation media with product will effectively

"depyrogenate" the media because any remaining endotoxin is unlikely to get into subsequent product unless some physical or chemical condition is changed.

ENDOTOXINS AS STANDARDS FOR THE LAL TEST

The term "endotoxin" was coined by Pfeiffer to distinguish a toxin that seemed to be an "integral" part of the bacterial body" from the endotoxin elaborated by the cholera bacilli (Westphal, 1993). The term is still in use today. It is used indiscriminately to refer to the naturally occurring wall fragments, to incompletely purified preparations of LPS that retain proteins and other components normally associated with LPS in the bacterial membrane, and to purified LPS preparations.

Methods of Purification

Several common methods to purify endotoxins are described in the literature. These include the classic trichloroacetic acid extraction of Boivin et al. (1933), which results in LPS still associated with membrane proteins; Westphal and Jann's (1965) phenol extraction, which results in recovery of high molecular weight LPS free of proteins; the phenol–chloroform–petroleum ether extraction developed by Galanos et al. (1969) for recovery of low molecular weight or R-form LPS; and a phenol extraction followed ultimately by electrodialysis to remove cations and enable manufacture of a highly purified uniform salt (Galanos et al., 1979b).

Reference Endotoxins

The endotoxin preparation that is the reference standard for the USP bacterial endotoxins test (USP, 1995a) is referred to as an reference standard endotoxin (RSE); it is a Westphal extracted preparation of endotoxin from cells of *Escherichia coli* O113 that is colyophilized with lactose and polyethylene glycol. The latest lot, designated lot G by USP and EC-6 by FDA, is a sublot of a large number of vials prepared at the National Institute for Biological Standards and Control in the United Kingdom. Two other sublets were pooled, and the second international standard (IS) was taken from these pooled sublets. The biological activity of lot G/FDA EC-6 and that of the second IS as measured by LAL are equivalent; 10,000 EU per vial = 10,000 IU per vial, where EU stands for endotoxin unit (a U.S. designation for LPS activity) and IU stands for international unit (a European designation for activity). The recent acceptance of this standard by the Expert Committee on Biological Standardization, World Health Organization, as the international standard has very important consequences for companies releasing product in the United States or Europe because it enables all laboratories to perform tests calibrated against the same reference.

Other purified endotoxins may be calibrated against the RSE (see the section on Potency Determination) and used to control the LAL test. These secondary standards are referred to as a control standard endotoxins (CSEs).

Relative Activity of Endotoxins

Since it is the amount of lipid A and its functional activity as determined by its bacterial source that correlate with biological activity, activity cannot be compared between

different endotoxin preparations on a weight basis. Endotoxin units, EUs or lUs, express the relative pyrogenic activity of an LPS preparation or unknown when tested with LAL. Originally defined for the Food and Drug Administration's endotoxin standard EC-2, the EU made it possible to compare any endotoxin preparation to the well-characterized standard in order to anticipate the preparation's pyrogenic potential. Once the activity of the preparation was standardized, it could be used instead of the RSE as the control standard in the LAL test. In the same way, the amount of endotoxin in an unknown must be expressed in endotoxin units per milliliter (EU/mL) so results, standardized to the activity of a known RSE, can be compared.

The LAL test has been approved by the U.S. FDA for 20 years now as the release test for medical devices and began replacing the rabbit pyrogen test in USP monographs in 1983. Today, the LAL test for gram-negative bacterial endotoxins has succeeded in replacing the rabbit pyrogen test in the majority of monographs, and most comparisons between the two have shown that the LAL test is an excellent predictor of pyrogenicity.

LIMULUS AMEBOCYTE LYSATE

The LAL reaction is an enzyme activation cascade that results in cleavage of a protein substrate. Under the right ionic conditions, the insoluble cleavage product coalesces with others to form a gel. As the pre-gel matrix grows, the particles become visible and the test solution becomes turbid. If an artificial, chromogenic, substrate is added to the reaction mixture, a chromophore will be cleaved that will cause the test solution to become colored. These three types of end points—gelation, turbidity, and intensity of color—define the three standard LAL test methods.

The LAL test is used worldwide as an end product release test for regulated products, and standard procedures are described in the various pharmacopoeias. In the United States, the manufacture of LAL reagent and the performance of LAL tests on pharmaceutical products are regulated by the Food and Drug Administration (FDA). The bacterial endotoxins test is the official method described in the U.S. Pharmacopeia (USP, 1995a). The test is modified with respect to assay validation and for routine release of end products by FDA in a guideline published in 1987 (FDA, 1987). This guideline, currently under revision (H. D. Hochstein, Center for Biologies Evaluation and Research of the FDA, personal communication), also describes the alternative methods, turbidimetric and chromogenic, that are not part of the pharmacopeial test. An FDA interim guidance publication (FDA, 1991) expands on the kinetic methodologies.

These documents, taken with the manufacturers' product inserts, provide the information necessary to perform LAL tests that would be acceptable to release product in the United States. The instructions for use are not exactly alike between manufacturers. Minor differences in the recommended test procedures and types of materials may be important for the success of the test, so it is important to follow the procedure described by the manufacturer of the LAL being used.

Gel-Clot Method

The gel-clot reagent was the first LAL reagent licensed, and the gel-clot method is still used for the majority of LAL tests. Equal volumes of sample and solubilized LAL are mixed in a $10 \times 75\,mm^2$ glass reaction tube. The mixture is incubated for 1 h at 37°C.

At the end of the incubation period, tests are read one at a time by removing an individual tube from the incubator (from a rack in a water bath or from a well in a dry-block heater) and inverting it in one smooth motion through 180°. If a gel has formed during the incubation and remains intact on inversion, then the test is positive, and the amount of endotoxin in the tube is greater than or equal to the labeled sensitivity of the LAL reagent. Any other state of the reaction mixture at the end of the incubation period constitutes a negative test.

Flocculant or "snowflake" precipitation is indicative of the presence of endotoxin in the reaction mixture but in an amount that is less than the sensitivity (see the section on "Turbidimetric Methods"). It is useful to note this precipitation when reading the test. If it occurs in the negative controls, it often means that the water source is contaminated with a low level of endotoxin. If low levels of endotoxin are added to the reagent when it is solubilized, the LAL reaction is initiated in the vial and the assay will appear to be more sensitive than it should be when standards are tested. Higher levels of endotoxin in the reagent will cause all the tests to be positive, including the negative control. If the gel matrix has started to form in the vial and is disrupted to set up the test, none of the tests will clot because the disrupted matrix will not gel again.

The sensitivity of the gel-clot method is limited by the time and temperature of incubation. The most sensitive reagent, defined by the 1 h incubation at 37°C, is able to detect as little as 0.03 EU/mL. The U.S. reference endotoxin is always used to establish the sensitivity of licensed reagents.

Turbidimetric Methods

As the LAL reaction progresses toward gelation, the reaction mixture becomes turbid. Those samples with greater concentrations of endotoxin become turbid more rapidly. Optical density, used as a measure of turbidity, may be read at the beginning of incubation and again at a given point in time after onset of incubation, in every sample. A standard curve may be drawn by plotting optical density (OD) against endotoxin concentration. This method of performing a turbidimetric assay is called an end-point method because samples are read at the end of a predetermined period of time.

The first descriptions of a turbidimetric method were given by Levin and Bang (1968). They performed what is termed a kinetic turbidimetric method because they took frequent OD readings in the same sample, and they showed that the samples with the greater endotoxin concentrations had greater rates of increase in turbidity than those with less endotoxin. Today, incubating spectrophotometers have been developed that will read OD values from multiple wells and read each sample multiple times, each reading made within seconds of the previous one. These values are transferred to a computer for storage and analysis. Calculations are performed, and the system returns endotoxin concentrations to the operator.

Kinetic methods lend themselves to screening samples that vary over a wide range of endotoxin concentrations, typically four to five orders of magnitude. The tests are performed by mixing solubilized LAL reagent and sample in whatever ratio is recommended by the manufacturer for the type of reaction vessel being used. Data are collected, and a standard curve is constructed by plotting the logarithm of the onset time against that of the endotoxin concentration. Onset time is the time at which the optical density reaches a predetermined value and is an indicator of the rate of the reaction. The standard curve has a negative slope because the onset time is fastest (lowest numerical value) for the greatest concentration of endotoxin.

Because turbidity can be detected before a reaction mixture clots, a given endotoxin concentration will yield a turbidimetric result sooner than it will yield a gel-clot result. Very low concentrations of endotoxin can be measured within a reasonable incubation time by turbidimetric means that would not be likely to cause a clot for several hours. The kinetic turbidimetric assay may be as sensitive as 0.001 EU/mL in a test that incubates for approximately 90 min.

The sensitivity of turbidimetric reagents, unlike that of the gel-clot reagents, is not determined by the manufacturer. The sensitivity is defined to be the lowest concentration used to construct the standard curve, so the analyst defines it and can change it to accommodate the interference characteristics of the sample and its endotoxin limit.

Chromogenic Methods

In 1977, Japanese investigators reported using synthetic or artificial substrates in the LAL assay (Nakamura et al., 1977). These substrates comprised a chromophore attached to a smaller number of amino acids in a structural sequence that mimicked the cleavage site of coagulogen, the naturally occurring LAL substrate. When activated clotting enzyme cleaved synthetic substrate, the chromophore was released. Thus, the amount of absorbance measured in the reaction mixture at any time was a function of the amount of chromophore released, which was in turn a function of the concentration of endotoxin in the sample.

As with the turbidimetric assay, the chromogenic assay may be performed using either an end-point or kinetic protocol, and the data are plotted and analyzed in the same way as for the turbidimetric method. Variants to the end-point method include use of diazotization chemistry to transform the typical yellow of the cleaved *para*-nitroanaline chromophore to a magenta. This is a useful step when performing the LAL test on samples that are already yellow.

The sensitivity of the chromogenic method is the least concentration of endotoxin included in the standard curve. The greatest sensitivity claimed for this method is 0.005 EU/mL.

Differences Between LAL Reagents

The formulations of currently available commercial LAL reagents differ from one manufacturer to another. These differences mean that some substances containing endotoxin will interfere with the sensitivity of one manufacturer's test more than with the sensitivity of another's (Twohy et al., 1983). Formulations may differ in their capacity to disaggregate or solubilize LPS particles to make a lipid A more accessible to LAL enzyme(s). Some formulations may stabilize the pH of reaction mixtures within the optimum range more efficiently or have components that adsorb LPS in such a way that lipid A regions are exposed.

β-Glucans

The LAL test was originally thought to be highly specific for lipopolysaccharides, but more recently it has been found to be sensitive to the presence of certain types of P-glucans, also referred to as LAL-reactive material or LRM (Kakinuma et al., 1981; Pearson et al., 1987; Roslansky and Novitsky, 1991). Factor G, an enzyme that is present in LAL, is activated by β-1,3-glucans or by β-l,4-glucans. The β-1,3-glucans are

components of fungal and algal cell walls; the β-l,4-glucans are common extractables from cellulosic materials. Formulations of LAL reagent differ in the level of sensitivity they have to the β-glucans; some are very sensitive, and others are practically insensitive. Endotoxins and β-glucans often act synergistically in an LAL test, so alterations to formulations that reduce sensitivity to the β-glucans may also reduce sensitivity to certain LPS structures (Roslansky and Novitsky, 1991).

Pharmaceutical products are rarely contaminated with β-glucans. Unless the product is derived from fungal or algal culture, these organisms and therefore the β-glucans are not found as contaminants in a facility that maintains good manufacturing practices. The most common source of cellulosic extractables is cellulosic filter media, but not all such media shed these polysaccharides. For example, chemically produced non-naturally derived cellulose acetate membranes are free of β-l,4-glucans. Filtrates that yield positive LAL tests indicate contaminated filter media whether the source is endotoxin or β-glucans. If the tests are negative, then β-glucans are not an issue. It is usually possible to exclude filter media suspected of excreting β-glucans from production processes. When it is not possible to change the type of filter medium, it is usually possible to rinse the medium before use to reduce the level of contamination.

A phenomenon noted in my laboratory and conveyed by others who have also looked at cellulosic materials is something referred to as "rebound," If a cellulosic substrate is rinsed until the effluent no longer causes a positive LAL test, stored for several hours under conditions that prevent bacterial growth, and then rinsed again with the same type of rinse solution as before, the second effluent will be positive or rebound if the original source of the LAL reactivity was LRM. The second effluent will be negative if the original source was endotoxin. Therefore, less LRM is likely to contaminate a product if the filter is rinsed with product and used immediately.

ANCILLARY LAL REAGENTS

The manufacturers of LAL reagents may supply ancillary reagents either separately or as kits. Ancillary reagents and materials that are required to perform the test are listed in the product insert or instructions for use.

To perform a satisfactory LAL test, one must have LAL reagent, an endotoxin control (CSE or RSE), and a source of water that has less endotoxin than is detectable by the type of LAL method used. Equipment includes reaction vessels (glass tubes or plastic microplates), a mixing device, dilution tubes, storage containers, pipetes and other such transfer devices, and closures. Equipment materials should not adsorb endotoxin or leach substances that interfere with the test.

Materials that come in contact with the sample or any of the reagents should be rendered, or shown to already be, free of detectable endotoxin and free of substances (extractables) that interfere with the LAL test. Materials that are labeled "sterile" may not be suitable for use, while and, on the other hand, materials that have not been sterilized may be perfectly acceptable. Materials labeled "nonpyrogenic" may still contain enough endotoxin to affect an LAL test even though they do not contain enough endotoxin to be considered pyrogenic. Medical devices are nonpyrogenic if the average amount of endotoxin detected per unit from a pooled sample is less than 20 EU (USP, 1995b; FDA, 1987). Polypropylene plastics have been particularly subject to endotoxin adsorption and to leaching of interfering substances (Novitsky et al., 1986).

ERROR OF THE LAL TEST

The error of the test currently depends on the method. Quantitation by the gel-clot method is performed by titrating the endotoxin to an end point (last positive test in a series of increasing dilutions of sample), so the error is plus or minus a dilution. The gel-clot assay is generally performed on serial twofold dilutions, so the commonly expressed error for the test is plus or minus twofold or 50–200% of the measured value.

The so-called quantitative assays, the turbidimetric and chromogenic LAL assays, are also performed on serial dilutions, but the measured response (optical density or onset time) of the unknown is taken from a standard curve derived from the responses of known endotoxin concentrations. It is possible to narrow the observed range of the error to something that approaches the ability of the LAL reagent to discriminate concentrations, i.e., reduce technician error to a minimum, but in reality manufacturers and the FDA recommend that the error of these tests be maintained within 25% for end-point assays and 50% for kinetic assays (FDA, 1991). Most recently, the Japanese Ministry of Health and Welfare has taken responsibility for writing a version of the bacterial endotoxins test in an effort to promote international harmonization. They propose a 50–200% error around the recovered value for kinetic methods in which endotoxins are quantified using a log—log relationship. Thus, in practice, there may be little real difference in the error of the LAL test no matter what method is used, and it is misleading to refer to the gel-clot method as qualitative and to the turbidimetric and chromogenic methods as quantitative.

CONFIRMATION THAT THE TEST WORKS PROPERLY

It is very important to confirm that the test is working satisfactorily within one's own laboratory by the use of controls, and this is addressed by the U.S. Pharma-copeial Convention and the FDA in the appropriate references. The sensitivity of the gel-clot lot should be obtained within the error of the test. The sensitivity of the turbidimetric and chromogenic methods must be demonstrated by achieving a linear relationship over a given range of endotoxin concentrations that is also sufficient to distinguish the concentrations from each other within the error of the test. The definition of linearity is an issue for the kinetic methods. Over broad ranges of endotoxin concentrations, it is very apparent that the points on the standard curve more closely resemble a curved line than a straight line. The log-log representation cannot eliminate the curvature completely, but linearity, as defined, is met if the absolute value of the correlation coefficient is less than or equal to 0.980, a relatively easy specification to obtain.

On close inspection of the data plotted as optical density versus time of incubation, it is obvious that as the concentration of endotoxin being assayed increases, the difference in time between the concentrations narrows until the ability to measure accurately at very short intervals of time becomes the limiting factor. So, even though linearity is confirmed, more precise measurements are made by working with lower concentrations of endotoxin and by limiting the range, two conditions that also virtually eliminate curvature as an issue.

SOFTWARE

Software used to analyze the data obtained by the kinetic methods should be well understood. Software manufacturers, whether the LAL manufacturer or otherwise, have

chosen ways to deal mathematically with the inherent kinetics of the LAL reaction. Questions may arise about how onset times are determined, how regressions are calculated to obtain standard curve parameters, whether or not threshold OD values should be varied during analysis, and how parameters that were used to validate products can be identified and stored, if necessary, to use for routine product release.

INSTRUMENTATION

More elaborate instrumentation is necessary for turbidimetric and chromogenic methods to incubate the samples during the reaction period and to read and record the OD values either directly to paper or to a computer so they are available for analysis. Computers are used to collect and store the variables of time and OD, perform the calculations to return the parameters of the standard curve, and calculate the concentrations of endotoxin in the unknowns.

Incubators are important to the performance of the LAL test. The LAL reaction is enzymatic; therefore, the rate of the reaction varies with the temperature of incubation. The test requires that multiple samples be incubated in the same device, so all reaction mixtures have to be maintained at the same temperature for the results of one test to be related to those of another. Incubators that accept tubes are relatively easy to validate with respect to the temperature in individual reaction mixtures. Microplate incubators are generally more difficult and sometimes impossible to monitor because thermistors are difficult to place or cannot be placed in individual reaction wells. The ultimate test for these systems is to perform the LAL assay on one concentration of endotoxin in all tubes or wells. By using standard parameters obtained from a previous test, concentrations may be assigned to the tests in each well. The results are assessed two ways in my laboratory. First, the concentration in each well is expected to be within 25% of the mean concentration recovered for all wells; second, even when all wells pass the 25% test, there is no tendency for concentrations to increase or decrease from one side of the plate to the other or from the center of the plate to the edges.

POTENCY AND RELATED ISSUES

The method used to determine the potency (EU/ng) of an LPS is described in both the USP bacterial endotoxins test (USP, 1995a) and the 1987 FDA guideline (FDA, 1987). The reference endotoxin is diluted and assayed in parallel with dilutions of the endotoxin preparation to be standardized. The sensitivity of the LAL expressed in endotoxin units per milliliter of reference endotoxin is equivalent to the sensitivity of the LAL expressed in units of mass per milliliter of the test preparation. The potency is the RSE sensitivity divided by the CSE sensitivity, generally expressed as endotoxin units per nanogram. As long as the activity of the RSE and CSE remain the same or are affected to the same extent by the solubilizing agent, the potency should remain the same. In general, the relative potencies do remain the same, a satisfying situation, because it is the basis of being able to use the LAL test to quantify endotoxins.

One of the more subtle issues to bear in mind during experimentation with endotoxins is the issue of enhancement and inhibition of the LAL test by the test solution. Enhancement is defined as the recovery of more endotoxin than expected; inhibition is the recovery of less endotoxin than expected. No matter which method is used, the initial

qualification of the LAL test method, or of the technician performing the LAL test, is performed on endotoxin suspended in water. Manufacturers label gel-clot formulations with the sensitivity determined by measuring endotoxin concentrations in water, and they characterize the turbidimetric and chromogenic methods using endotoxin in water. When endotoxin is suspended in solutions other than pure water, it may assume a configuration that is more or less reactive in a biological assay, including the LAL test. The level of enhancement or inhibition that results can be thought of as a change in potency between the endotoxin in water and the endotoxin in the test solution. Enhancement or inhibition may be overcome by sufficient dilution with water to ensure that the endotoxin is, in fact, in water and no longer influenced by the test solution.

The presence of enhancement or inhibition is demonstrated by performing the LAL test in parallel on endotoxin concentrations prepared in pure water and on endotoxin concentrations prepared in the test solution. Using the gel-clot method, one shows that the sensitivity of the LAL to endotoxin in the test sample is the same as in water, that is, the labeled sensitivity of the LAL is recovered in each test. To perform this test by the gel-clot method, the sample cannot contain detectable endotoxin because the test cannot quantify less than the concentration that causes a clot to form. The inhibition/enhancement test can be performed on samples that already contain measurable endotoxin only with the turbidimetric and chromogenic methods, with which endotoxin may be quantified by reference to a standard curve. The recovered additional or "spiked" endotoxin is distinguished from the endogenous endotoxin by subtracting the amount recovered in the sample from the amount recovered in the spiked sample.

If kinetic methods are used and several concentrations of endotoxin have been used to spike the sample, two standard curves are generated: the standard curve in water and the standard curve prepared in a single concentration of sample. These two curves on a log—log plot should have identical slopes, showing that the endotoxin activity dilutes in the same way for both preparations. If there is no enhancement or inhibition, the lines will overlie each other. If there is either enhancement or inhibition, then the vertical distance between the two lines is a measure of the difference in activity. If the slopes are not identical, then one line will cross the other, indicating that the sample enhances the activity of the endotoxin relative to water on one side of the crossover point and inhibits it on the other side. When the slopes are not identical, the concentration of endotoxin in the sample cannot be calculated by comparison to the activity of concentrations in water. The sample must be diluted further to a concentration that more closely resembles water and the test repeated (Remillard et al., 1987).

Obviously, the usefulness of dilution is limited for many experimental applications because the activity of the endotoxin is of interest only in the more concentrated sample. In these situations, there are two choices. If the sample can be rendered free of detectable endotoxin, the standard curve is prepared by making dilutions of RSE (or CSE that has been calibrated in the sample against RSE) in sample that isfree of endogenous endotoxin. This "product standard curve" can then be used to quantify endotoxin in the unknown. The parameters of the standard curve should be shown to be constant between several samples before one sample is used to prepare standard curves for other unknown samples. One application for which it is inappropriate to use one sample to prepare standard curves is in the measurement of endotoxin in blood, because activity of endotoxin detected by LAL is a function of the neutralizing capacity of the individual source of the blood as well as the amount of endotoxin in the sample (Warren et al., 1985; Novitsky, 1994).

The second method used to measure endotoxin in a sample concentration that otherwise interferes with the LAL test is to remove the endotoxin from the sample and

restore it to a water solution before performing the assay. This has the advantage of putting the endotoxin in the same background, and presumably in the same active configuration, so that concentrations obtained for endotoxin from the different types of samples can be compared to the activity of the standard endotoxin in water. Most methods that may be devised to quantitatively transfer endotoxin in low concentrations from one type of solution to another do not work. The greatest impediment to these procedures is the tendency of endotoxin to adsorb, and always to some degree adsorb irreversibly, to surfaces. This occurs when solutions are evaporated and the residue is resolubilized in water or when solutions are filtered and the material is resuspended in solution.

REMOVAL OF LAL BY FILTRATION

There are very few filtration materials that do not adsorb endotoxin; therefore, most are useless for recovering small concentrations of endotoxin. One commercially available ultrafiltration system that does work is specifically advertised to enable the user to remove low molecular weight interfering factors from LAL samples while retaining the higher molecular weight endotoxins in the retentate. It is basically a diafiltration system that replaces the sample solute with water; the sample (retentate) can be adjusted to the original volume at the end of the process and assayed directly. As long as the ultrafilter remains wet, endotoxins do not bind; and as long as the endotoxins are maintained in high molecular weight structures so that they are retained, they will be recovered completely in the assay.

This method works only if endotoxin can be retained in a high molecular weight form and if the solute is of a low enough molecular weight to be removed. If the solute causes endotoxin to disaggregate, the endotoxin will be lost with the low molecular weight substances in the filtrate.

This diafiltration method is extremely valuable if products that are insoluble in water are soluble in another solvent that is compatible with the filtration membrane and cartridge. Product can be solubilized in the solvent and diafiltered with the solvent to remove the low molecular weight product and retain the high molecular weight endotoxin. Once product is removed, the solvent can be replaced with water and the retained endotoxin tested.

This method has also been used in my laboratory to concentrate the endotoxin in samples. Some medical devices, for example, are so large that the rinse volume necessary to extract the contaminating endotoxin dilutes the endotoxin beyond the limit of detection (USP, 1995b; FDA, 1987). If the system is modified to ultrafilter several known volumes of sample, the retentate represents a concentration of the extraction volume and of the endotoxin, so an LAL test can be used.

Filtration is often used in a production process to remove endotoxin (depyrogenate). While there are some general considerations useful in choosing the type of filter medium and size or molecular weight cutoff, the only ultimate way to be assured that the filtration works is to use it successfully.

Sterilizing filters will not exclude passage of endotoxins on the basis of size but are successfully used to nonspecifically adsorb endotoxins. These filters "work" until the surfaces are saturated with endotoxin; therefore their use requires monitoring to ensure that endotoxins do not "break through." Positively charged filters often are more efficient at adsorbing negatively charged endotoxins. Adsorption, whether facilitated by surface hydrophobicity or by charge, works most efficiently when the nature of the solute

promotes adsorption and does not block it. Water and saline solutions generally facilitate adsorption.

Ultrafilters retain endotoxins as long as the endotoxins are large enough to be excluded by the pores of the membrane. If the molecular weights of product and endotoxin are different enough for one to be retained, then ultrafiltration can be used to separate the endotoxin from the product. Ultrafilters rated to retain particles that have molecular weights of 100,000 or greater will be perfectly adequate to remove endotoxin from water where endotoxins are expected to be in highly aggregated states. On the other hand, if the solute disaggregates endotoxins, then smaller pore diameters will be required to retain the endotoxin (Sweadner et al., 1977). If the product itself adsorbs endotoxin, then filtration will not separate them.

The aggregate state of the endotoxin is not always known before filtration is attempted. Some solutes seem to have somewhat surprising effects; for example, Tris ion competes with other cations for specific cation-binding sites on the LPS molecule and apparently facilitates solubilization (disaggregation) of the bacterial outer membrane (Schindler and Osborn, 1979). Consequently, it is relatively difficult to remove endotoxins from Tris buffers by ultrafiltration.

CONCLUSION

The LAL test has been developed and works very well to estimate the concentrations of gram-negative bacterial endotoxins in samples. The test is used to monitor production processes and release finished product. It is used to measure the efficiency of endotoxin removal by depyrogenation methods. It is used to help elucidate the biological activity of endotoxins as they are moved from one chemical environment to another. It can be used to detect inadvertent changes to formulations, especially changes introduced by β-glucan contamination, by responding with an uncharacteristic inhibition/enhancement pattern. In fact, LAL is a very useful tool with broad applications for the technician who takes the time to understand both endotoxins and LAL methodology.

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Media and Buffer Filtration Implications

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INTRODUCTION

As filtration applications have evolved, so have filtration purposes and designs changed. Filter systems are no longer standardized and limited to a tight filter portfolio, but broadened with new requirements and needs by emerging applications. Such filter designs experienced optimization, geared to meet the specific process parameters required for such applications. Two major applications, which differ greatly from each other, will be described within this chapter, and the specifics and design needs of these two applications will also be explained. Media filtration, one of the two applications, commonly focuses on a filter design that creates the highest total throughput and lowest fouling, influenced by unspecific adsorption. Buffer filtration with low fouling or blocking potentials is commonly used with high flow applications. The processing of buffer batches need to be fast and efficient, and therefore the design of the filter system is very different than the one used in media filtration.

Potential parameters which determine specific design criteria are:

- flow rate,
- total throughput,
- unspecific adsorption,
- retention rating,
- thermal and mechanical robustness,
- chemical compatibility,
- extractable/leachables,
- hold-up volume, and
- disposability.

All criteria have been considered and sterilizing grade filters evolved to high performance, meeting biopharmaceutical and regulatory requirements. New welding and membrane treatment technologies reduced the extractable/leachable content greatly. Wetting agents used formerly are no longer required due to enhanced membrane surface treatments. New polymeric developments and membrane structures create the possibilities of higher thermal and mechanical resistance. Support fleeces that sandwich the membrane

form a higher stability. Fleece choices and developments, as well as membrane enhancements, have resulted in improved flow rates and total throughputs. Comparing some of the performance data of 20 years ago, filter cartridges of high flow rate designs now reach 5 times higher flow rates than former counterparts, and total throughput increases reach a 3 to 4 times higher level. All these improvements were needed to be able to fulfill a growing stringency by specific applications. Furthermore, it created the opportunity for the end-users to optimize their processes, cut down costs, or enhance production yields (Soelkner et al., 1998; Jornitz et al., 2002, 2003, 2005; Cardona and Inseal, 2006).

MEDIA FILTRATION

Application Considerations

Growth media are widely used in the biotech industry, for example, for microbiological tests, cell culture and fermentation processes, or media fill testing. The major volume use for media is within cell culture and fermentation processes, which will be the focus of this chapter. Growth media of the past were commonly standardized and animal derived. Since cell lines have become more and more specialized, the media followed suit and nowadays over 60% of the media used are special media, which are developed for a specific cell lines and processes. These recipes are highly guarded secrets as the media is a major component in determining cell density, performance, and downstream processing activities. Any change within the recipe of a specialty media can potentially influence the drug product outcome. This also means that filtration devices used within such specific media filtration applications should not be influential in regard to the media composition, that is, neither adsorb a particular component to the membrane nor leach an extractable into the media. Appropriate tests have to be performed to determine whether such possibilities exist with the device and membrane polymer used. Process validation work is a key element to establish whether or not a filter fulfills the required performance parameters. Such validation work starts with the media supplier and the filtration steps within their production processes and ends with an end-user validation to manifest the filter performance in the end-users environment.

The incidences of transmissible spongiform encephalitis caused the industry to take precautious measurements, especially tracing the raw material back to the origin of the herd. Still, with pooling, batch-to-batch quality variations and difficulties of traceability, the media users started switching to plant-derived media (plant peptones). But this switch caused another problem, which has become a widely discussed issue, *Mycoplasma* contamination of the media. *Mycoplasma* contaminations are on the rise and require attention. It is very difficult to detect *Mycoplasma* contaminants, as well as difficult to remove them. Most commonly 0.1 μm-rated filters are used to remove a potential *Mycoplasma* contamination, however it is not and will not be the "silver bullet," that is, process validation of the particular separation process is essential to assure retentive effectiveness. The advice is probably to use a multi-step removal/inactivation process to be completely assured that the contamination is eliminated. A recently formed Parenteral Drug Association (PDA) task force is working on a multi-facet solution of this issue, which includes novel detection methodologies, inactivation steps, and removal by filtration.

Furthermore, growth efficiency within plant-derived media might not be as effective as with animal-derived media. For this reason growth promoters are added, for example insulin or insulin-like growth factor (IGF) (Yandell et al., 2004). The growth promoters play an essential role and are a critical component of the media used. Therefore

any unspecific adsorption of the IGF on the membrane polymer would be detrimental to the media's quality and the cell culture growth. Once more the filter choice by testing for such unspecific adsorption is of importance.

Specific Requirements

Media are available in a large variety of different raw material sources and different compositions. Additionally the raw material quality experiences seasonal, dietary, growth, and regional variations, which sometimes makes it difficult to define the exact performance of a raw material. This factor can be challenging when filtration systems have to be determined and sized. Therefore, the main performance criterion for filtration systems for media is total throughput or filter capacity, the total amount of fluid which can be filtered through a specified filtration area. Filters used in media filtration are required to be optimized to achieve the highest total throughput and will be tested accordingly. To achieve reliable data, it is always advantageous when the test batch is at the lower end of the quality specification to gain a worst case scenario. Temperature, differential pressure, and pretreatment of the filter play an important role in performance enhancement of the filter system (Meltzer, 1987; Jornitz and Meltzer, 2001). For example, it has been determined that lowering the temperature of the media to be filtered and even the filter system might enhance the total throughput by 30%. The flow rate will be affected by the higher viscosity, but again the essential performance part is not flow, but total throughput. Flow rates in the filtration of biological solutions that were too high showed the negative side effect of gel formation on the membrane and therefore premature blockage. Starting with lower differential pressure has been seen to be advantageous, as again gel formation and/or cake compaction will be avoided. The lower the differential pressure at the start of the filtration, the better the performance. A preflush of the filter system, with preferably a cold buffer, will also enhance the total throughput. Hitting the filter with just the media has been found to foul the filter faster and therefore reduce the filter's capacity. In instances it is necessary to utilize prefiltration combinations to avoid fouling or blocking of the sterilizing grade or 0.1 μm final filter element. These combinations need to be determined in filterability trials to gain the most optimal combination to filter the particular media and to size the system appropriately (Fig. 1).

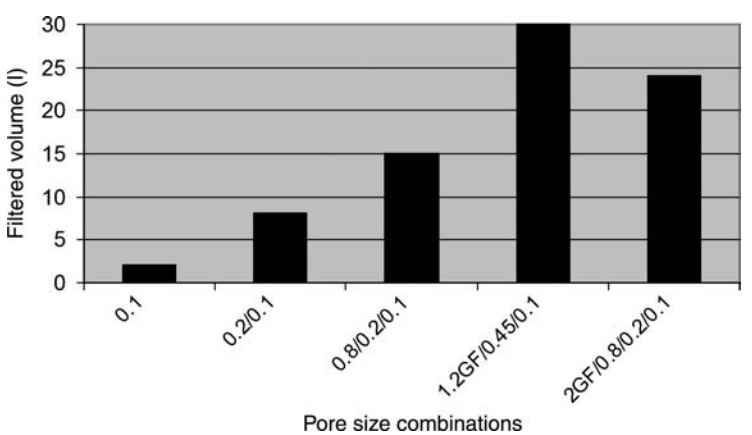


FIGURE 1 Total throughput determination of different pore size combinations.

Another important but often overlooked factor of media filtration is the influence of unspecific adsorption of the filter material. To separate lipids in the media, raw material adsorptive filter media are desired. However, in cell culture media, especially those containing growth promoters, unspecific adsorption has to be avoided. Certain membrane polymers do have a higher unspecific adsorption (Fig. 2). Sometimes the membrane polymer can be of similar type, but the surface treatment of the polymer is different or the design of the filter device is different. In any case, high unspecific adsorption can have an influence on growth promoters like IGF.

A higher unspecific adsorption of the polymeric membrane material also has an influence on the total throughput performance of the filter. The higher the unspecific adsorption, the higher the fouling of the filter. Accelerated fouling will result in a lower filtered volume through the filter device (Fig. 3). Often the fouling of the membrane will also result in difficulties to integrity tests post-filtration. Copious amounts of water are required to flush the filter to achieve either complete wetting of the membrane or elimination of product residue, which would negatively influence the integrity test. The last resort in these instances is to flush the filter with a solvent or a solvent/water mixture and perform the integrity test afterwards.

Adsorptive removal of a constituent of the media is one factor that could hinder cell growth. Another factor is release of leachables/extractables into the media. Extractables released from either the filter or other polymeric equipment could inhibit cell growth (Cahn, 1967; Jornitz, 2002). Filter devices these days have a very low extractable level in the ppm range and commonly do not pose a risk (Reif, 1998). However, media filtration happens also with gamma-irradiated, pre-sterilized filter/holding bag assemblies. Since gamma irradiation can have degradative effects an increase in an extractable might happen. This factor has to be considered and evaluated. Some studies showed a decrease in pH due to an acetic acid increase by polymeric degradation. Whether this is the case with the disposable system used and what effect this pH shift has must be evaluated. The range of any pH increase certainly depends on the fluid volume–disposable system surface ratio. The smaller the fluid volume, the higher a pH shift might be. In most scenarios, however, the vendor's awareness resolved such issues and more applicable

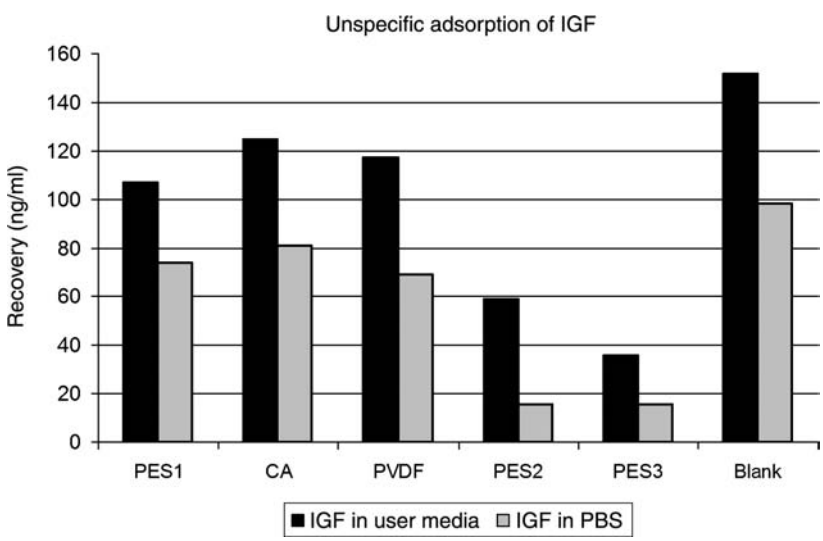


FIGURE 2 Unspecific adsorption of IGF to different filter devices.

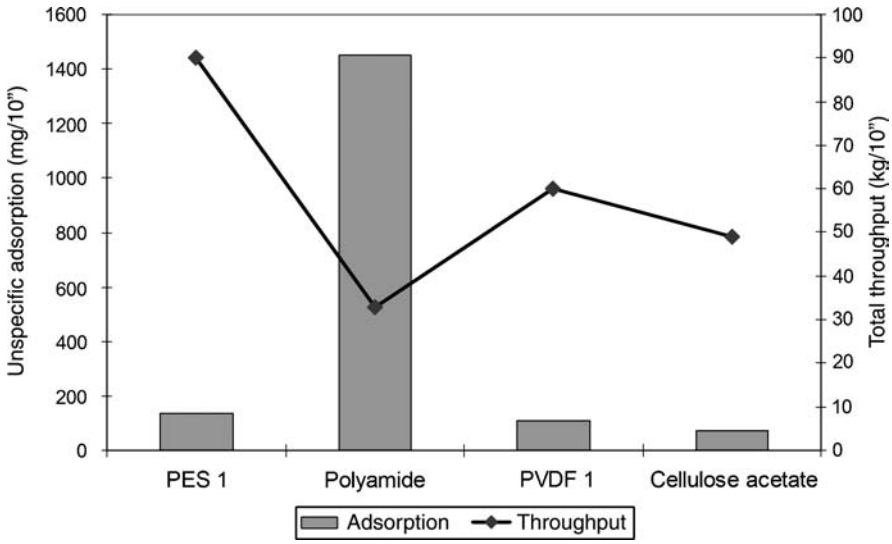


FIGURE 3 Influence of higher unspecific adsorption to performance.

polymeric materials and film to be used in media application was developed. Process validation, which includes extractable studies, will verify that the filter–holding vessel assembly fulfills the set specification.

As described, *Mycoplasma* contaminations are no longer a problematic topic of the past, but with the switch to plant-derived media, have become a current issue that must be addressed. One possibility is to filter the media through a 0.1 µm rated filter. Nevertheless, one has to be aware of the fact that there is no such standard as the ASTM 838-05 (2005) 0.2 µm rated filter challenge standard for 0.1 µm rated filters. Therefore one will find varying retention capabilities of such filters (Fig. 4). The *Mycoplasma* retentivity of a 0.1 µm filter does not only depend on the pore size distribution, but also on the filter construction and thickness of the membrane. A double layer membrane system reduces the bioburden toward the final filter membrane and

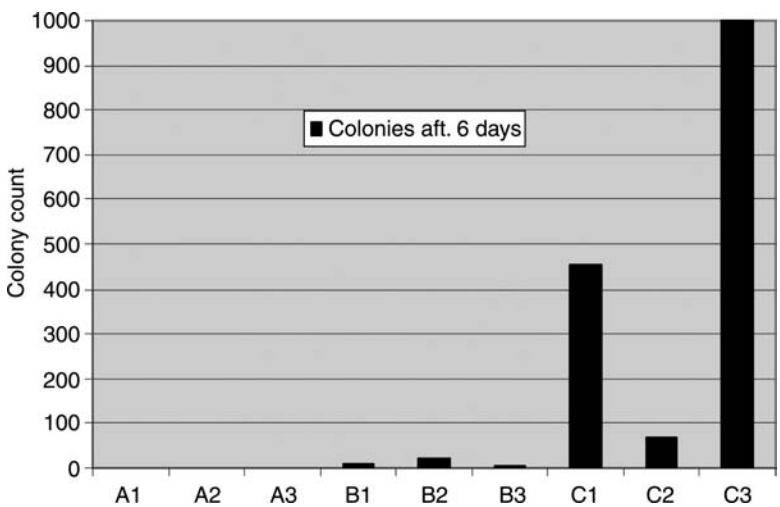


FIGURE 4 *Mycoplasma* retentivity of different 0.1 µm rated filters at same challenge conditions.

commonly shows a higher retentivity. Tests have shown that at high bioburden level challenges, organisms were found on the filtrate side. The likelihood of a *Mycoplasma* penetration increases with decreasing membrane thickness. It is advisable to utilize a sufficiently thick *Mycoplasma* retentive membrane layer, instead of aiming for improved flow rates and thinner membrane layers. Process conditions have a high influence on the separation of the organism. Higher pressure conditions or pressure pulsations might support *Mycoplasma* penetration.

Mycoplasma removal by filtration with 0.1 μm rated filters is one possible step within a process. Nevertheless, since the issue is as critical as viral removal, a process should have multiple removal/inactivation steps and filtration should be supported by other means like heat treatment. An advancement in 0.1 μm filtration will be a proposed standard challenge test, which will be designed by a PDA task force. This test should be forthcoming by late 2008 and would help to finally be able to compare the commercially available 0.1 μm rated filters. The end-user however has to be encouraged to perform process validation to verify that the filter performs as expected within the end-users process. Process conditions require testing, as laboratory conditions can only be an indication.

Filter Designs

Total throughput, the measure of the total amount of filtrate that can be filtered before the filter element blocks, is probably the most widely required performance criteria in most of the applications, but specifically in media filtration. It is directly proportional to the filter surface area, system size, and prefilter combinations. Certainly the impact on the total filtration costs can be substantial if the filter is not optimized for total throughput or an inappropriate pre- and final filter combination is used. What may appear to be a less expensive filter could actually significantly increase the filtration costs.

The total throughput of a filter cartridge depends on the membrane filter polymer, pore structure, and filter design. As previously elaborated, some membrane polymers are highly adsorptive, which might be of use in specific applications. Nevertheless, higher adsorptivity is commonly associated with a higher fouling rate and therefore lower total throughput. Total throughput optimized filter element commonly utilize a membrane polymer which is either moderate or low adsorptive.

Some membranes have a higher asymmetric proportion, that is, a larger pore structure on the up-stream side becoming finer throughout the membrane to the filtrate side (Fig. 5). The overall result, in effect, is an assembly of "V" shaped pores. The filter

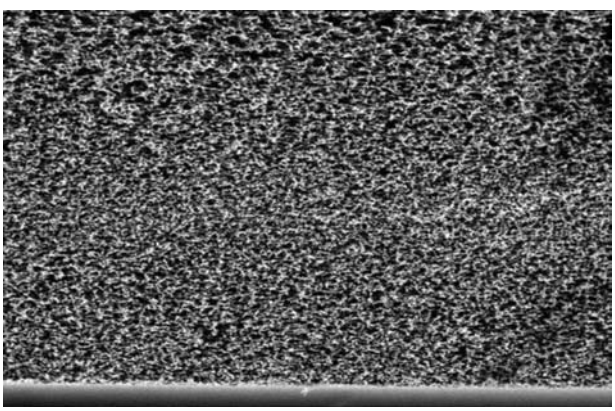


FIGURE 5 Highly asymmetric polyethersulfone membrane.

cartridges are so constructed that the more open ends of the "V" shaped pores of the membrane are directed upstream. This enables them to accommodate larger deposits in their more open regions. The result is a commonly larger total throughput than a symmetric membrane structure. Another side effect which asymmetric membranes showed is a higher flow rate, which might be a result of a lower resistivity.

Another design improvement is the membrane combination within the filter element. A coarser prefilter membrane layer in front of the final filter membrane, the so-called heterogeneous double-layer membrane, has a distinctly higher total throughput due to the fractionate retention of the contaminants (Fig. 6). The pre-filter membrane, for example 0.45 μm rated, creates a protective layer over the 0.2 μm rated final filter membrane. A large load of contaminants are removed first before they reach the final filter membrane. This fact is similar to the utilization of pre-filter combination in front of the final filter cartridge, except the pre-filter membrane of a heterogeneous double layer construct is contained within the filter cartridge element. This factor also avoids the need of two separate filter housings, if one wants to use separate retention ratings within the filtration process. Certainly the pre-filter layer within a heterogeneous filter design adds to flow resistance, therefore the flow rate of a heterogeneous filter design is lower than its single layer counterpart, but higher than a homogeneous (0.2/0.2 μm) filter cartridge. Flow, however, is not the main performance criterion within media filtration. High throughputs are sought.

Further parameters to improve the total throughput within a filter cartridge design are the choices of prefilter fleeces and the effective filtration area (EFA), that is, the pleat design of the membrane within a filter element. Unique pleat designs can create a higher filtration area within the filter cartridge and therefore higher total throughputs (Soelkner et al., 1998; Cardona et al., 2004). Thinner pre-filter fleeces allow higher pleat amounts within a filter element. However, if these are too thin, the flow channel between the pleats potentially may be too tight and a complete filtration area utilization might not be given. A thicker fleece creates a wider flow channel and also protects the next filter layer. Nevertheless, thicker fleece constructs restrict pleat density and also might press into the membrane. A well administered balance of construction criteria is

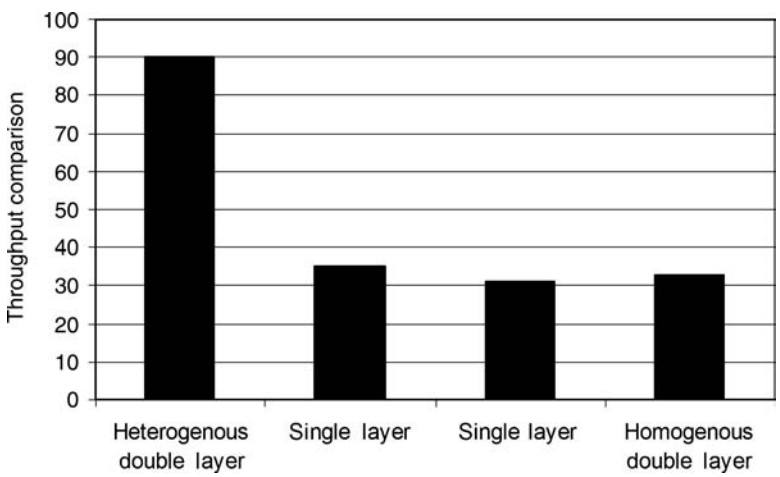


FIGURE 6 Total throughput differences of 0.2 μm rated filters with different membrane combinations.

required to be able to design a high performance filter. Vendors have optimized the filter design accordingly.

All these construction parameters result in optimal total throughput performance and certainly differ from filter to filter (Fig. 7). Such performance differences can even increase depending on the fluid to be filtered. Finding the optimal filter combination can mean potential savings of 50% of the total filter expenditure in the specific application or hundreds of thousand of dollars per annum.

The total throughput can be further advanced by evaluations of appropriate pre- and final filter combinations, if required. A lower cost prefilter might be used to protect the final filter and reduce the required final filter size.

Testing and Sizing Filters

Total throughput tests to determine the appropriate final filter and/or combination of pre- and final filter are performed with 47-mm flat filter composites. These composites have to have the same fleece and filter combination as the filter element to be used later. Commonly, multiple composites are tested to determine the appropriate final filter and to be able to test multiple prefilter options. These tests will determine the optimal combination that achieves the highest fluid throughput per EFA (Fig. 8).

When 47-mm discs are used, venting of the test filter system is essential. If the filter device is not vented appropriately, the entire EFA is not used and the test result will be false (Fig. 9). If, and unfortunately it does happen, the results of a 47-mm trial are used to size the required filter system, the system would be greatly oversized.

Often filter combinations of different filter vendors are tested to establish the best possible filter combination and to potentially determine a second supply source. Such comparison tests need to be designed carefully, so that the comparison is truly effective and not skewed due to wrong information. For example, 47-mm disc tests can be either performed by using a stainless steel filter holder or a test disposable device. If this is not

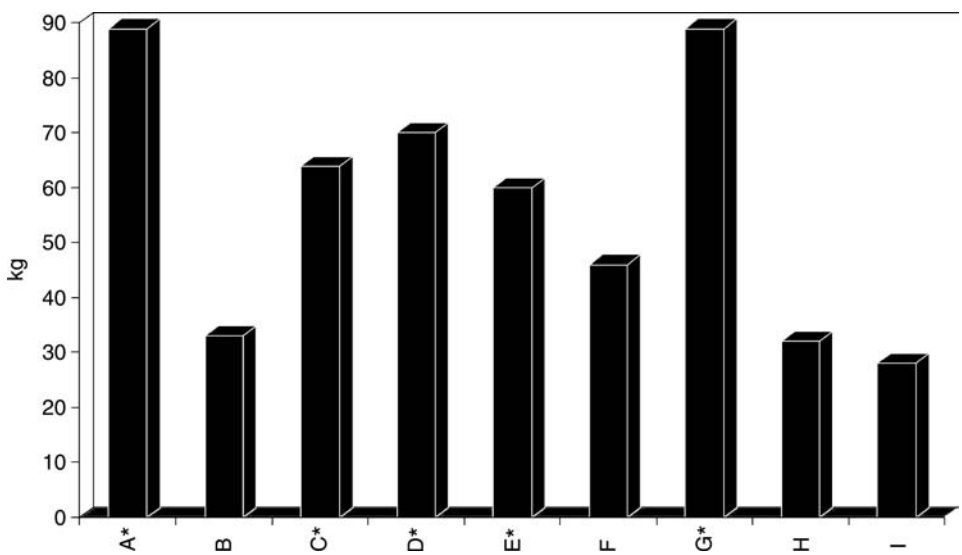


FIGURE 7 Total throughput comparison of different 10-inch polyethersulfone filters and 0.2 μm sterilizing grade membrane filters using a model solution (* indicates a double layer filter).

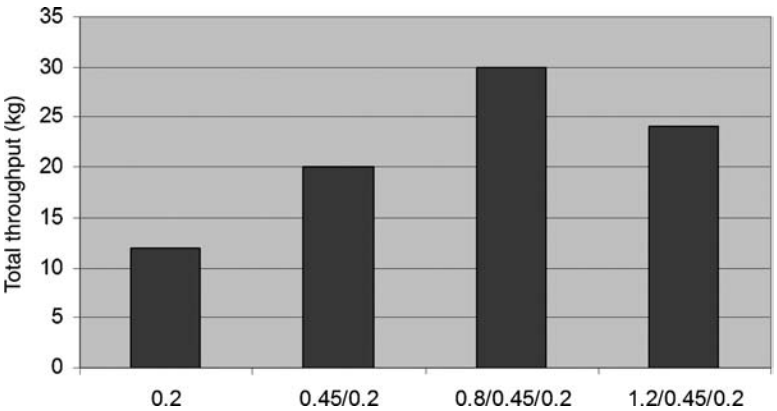


FIGURE 8 Total throughput comparison of different 47-mm flat disc pore size combinations using a model solution.

followed, the tester may believe that the filtration area of these two very different devices is the same and may even have been informed as such by the vendor's technical literature. However this is not the case, as the disposable test device commonly has a 36% larger filtration area (Fig. 10). The tester would have thought that the throughput of the filter membrane combination within the disposable device is far superior than the 47-mm filter composite in the stainless steel holder, not realizing that the performance of the disposable filter might be worse and only performs better due to the larger surface area. For that reason filterability tests require precise procedural definitions, information by the vendor, and interpretation of the test results.



FIGURE 9 A 47-mm test filter that was incorrectly vented.

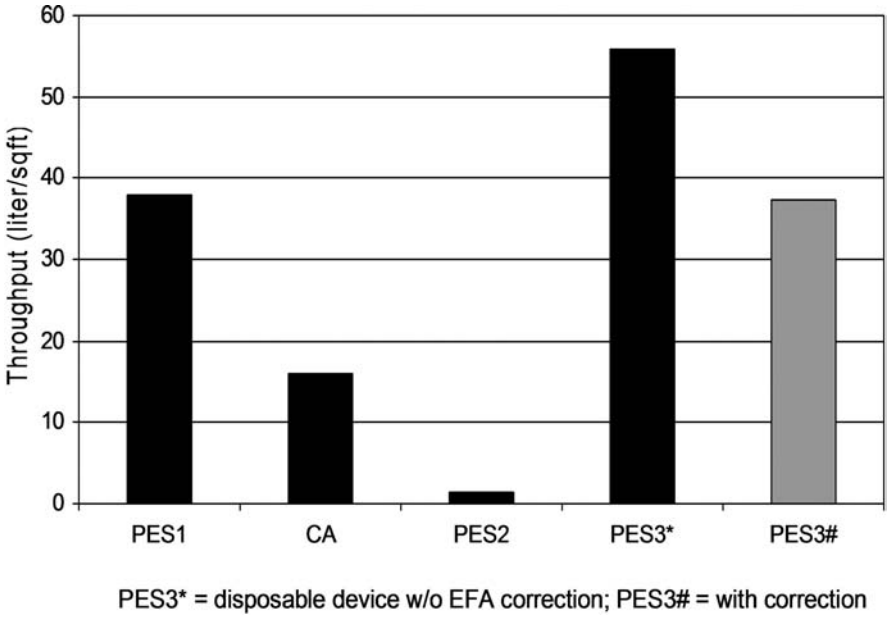


FIGURE 10 Filterability results of different filter materials, one filter device being disposable with a larger surface area (PES 3*).

If there is sensitivity to unspecific adsorption, these small scale trials can be utilized to sample the filtrate and determine any yield, activity, or constituent loss. Samples of the filtrate would be taken at frequent, pre-specified intervals and assayed for eventual losses or filtrate alterations. These trials can determine the losses, which might occur in process scale, and therefore allow counter measures to be taken. Besides, during the trial work with 47-mm discs, optimal processing conditions such as differential pressure or temperature and pretreatment protocols, or appropriate buffer flushing, can be determined. It should be noted that stand-alone 47-mm adsorption studies may be of limited utility. Comparison of the membranes under consideration under identical conditions is recommended.

Yet, 47-mm tests can only suggest the best filter combination, that is, serve as an indicator trial. To define the proper filter size required within the production process, small scale pleated devices of the predetermined filter combination should be utilized. These tests are utilized to verify the findings of the 47-mm disc trails. Only these pleated devices offer linear scalability as these are of the same design as the large-scale filter elements. It is necessary that the design of such filter devices be an exact replicate of the final combination that will be validated in the full-scale process, otherwise linear scalability is impossible. Test data collected with 47-mm discs or during small-scale filter device evaluations might be used within a database system for future filter performance choices. The common procedure of testing starts with an indicator trial (47-mm discs) to find the best filter combination, followed by verification trials (pleated small scale devices), which verify the findings of the indicator trials and create a possibility of sizing, and followed by confirmation trials (process scale) which validate the filter choice and size made in the verification trials. When this procedure is utilized the end-user can be assured that the filtration system is optimally tailored for the application's needs.

Validation Needs

The filter vendors test any distributed filter element or device within their laboratories to specific standards, such as USP. These tests encompass a multitude of tests which are required by regulatory authorities, but also additional tests to determine stability specification for the filter element. In the past, tests performed by the filter vendors were utilized as validation tests or data; however, because the tests are performed within laboratory settings under standard conditions, they can only be viewed as qualification tests. The vendor qualifies that the filter developed meets the defined specifications and stays within these specifications repeatedly and reliably. Commonly qualification trials are comprises of the following tests:

- water flow rate
- size/dimensions
- temperature/pressure limits
- steamability
- correlation of the integrity test values to bacteria retention
- chemical compatibility
- extractables
- endotoxin
- particulates
- biosafety
- pH/conductivity
- oxidizable substances
- for 0.1 μm rated filters, *Mycoplasma* retentivity

These test data are made available by the vendors in qualification documentation. Within this documentation the vendors are also adding other essential technical data, as well as item numbers, quality certificates, and product descriptions.

Since the data determined by the filter vendor are run at standard conditions, the end-user requires performing process validation work, evaluating the filter device performance within the process environment under process conditions. Specific tests are described with guidelines (FDA, 2004; ISO, 2003), which require to be performed to verify the filters performance within the production environment. These tests can include following activities:

- viability testing
- bacteria challenge testing
- extractable/leachable testing
- particulate testing
- chemical compatibility testing

All tests should be performed under process conditions and with either the actual drug product, in this case media, or a placebo or model solution. The main reason to perform these tests is to verify that the filter performs as expected within the process. Filterability trials described previously are the first step to establish the optimal filter combination. When performed under process conditions, these tests allow compatibility, extractables and particulate matter to be tested. Compatibility especially requires immediate determination, as it is a knock-out criterion. However, the next immediate steps need to be challenge testing to evaluate that the filters retentivity is not compromised by either influences of the separation mechanisms, membrane stability, or organism structure. For example, could high ionic strength of the solution potentially shrink the organism and cause penetration of a

filter device? *Mycoplasma* challenge tests are important to verify the filters retention performance with the media used instead of a standard challenge solution and standard conditions. The process fluids and parameters can have a unfavorable impact on the filters performance. If so, such incidence needs to be determined.

BUFFER FILTRATION

Applications

The variety of buffers and their applications are limitless (Table 1). However most use of buffers is for pH adjustment within the cell culture process or elution promoter or isoelectric point adjustment within the purification process. In cell culture processes the buffer utilized to adjust the culture's pH consists most often of a basic buffer (glutamate, sodium carbonate) or acidic buffer (citric-, malic-, acetic-acid). The buffer is fed automatically into the cell culture or fermentation process to keep the pH level at an optimal setting for cell proliferation. The pH might also be of importance for extracellular product stability and therefore has to be considered within the development of a process. Strong pH adjustments are also used in viral inactivation within the downstream process. The process fluid is adjusted to a pH of 3.5 to 4 for 30 min at ambient temperature. The mixing during this process time is essential to achieve unified pH adjustment within the set timeframe. Certainly the appropriate pH specification, time, and temperature depend on the virus to be inactivated and the product itself. Process validation studies will determine which setting would be optimal. To stabilize products in the down-stream process, for example diafiltration, phosphate buffers find their main use. Buffers function not only as pH adjustment, but also as adjustment of the ionic strength of the solution. In individual process steps it is essential to either lower or eliminate the salt concentration within the fluid. pH as well as salt concentration gradients are main functions for chromatographic capture and elution. The fine tuned-balance established during the development of the process will be mainly adjusted by specific buffers used in the individual processing step. In some instances buffers are also used to pre-wet or flush either filtration systems or equipment to avoid potential product alterations, degradation or precipitation.

Buffers play an essential role in the aforementioned process steps and can be used in large volumes. Since buffers come in contact with the product or are introduced into the cell culture vessel, the buffers must be sterile to avoid any contamination of the

TABLE 1 List of Common Buffers

Industrial term	Chemical name	pK_a at 25 °C	Buffer range
Acetate		4.76	3.8–5.8
Bicine	N,N-bis(2-hydroxyethyl)glycine	8.35	7.6–9.0
HEPES	4–2-hydroxyethyl-1-piperazineethanesulfonic acid	7.48	6.8–8.2
MES	2-(N-morpholino)ethansulfonic acid	6.15	6.1–7.5
PIPES	Piperazine-N,N-bis(2-ethanesulfonic acid)	6.76	6.1–7.5
TAPS	3-{[tris(hydroxymethyl)methyl]amino} propansulfonic acid	8.43	7.7–9.1
Tricine	N-tris(hydroxymethyl)methylglycine	8.05	7.4–8.8
Tris	Tris(hydroxymethyl)methylamine	8.06	7.5–9.0

culture or process. Buffer filtration with sterilizing grade filters is probably one of the largest filter consuming process steps, since the buffer volumes required are substantial.

Specific Requirements

As described, buffers are employed in every step of a biopharmaceutical process. Whether considered critical, for example in the formulation process, or not does not matter; the buffer still requires to be filtered, most often with a sterilizing grade filter. The filtration step will eliminate any impurity or contaminant, which otherwise would enter the process chain. If the contaminant is a microorganism, it might proliferate within the process step and cause either biofilm or endotoxin problems in future.

Filtration of buffers happens most commonly from a mixing vessel into a holding vessel. The holding vessel is then stored within a cold room till buffer use. Stainless steel vessel systems are nowadays more and more replaced by disposable mixing vessels and holding bags (Fig. 11). The holding bags can also be designed in a manifold fashion so the bag volumes are easy to handle and are available as required (Fig. 12). Disposable devices have the benefit that these are gamma irradiated, are ready-to-use, do not need to be cleaned, and are sealed systems. The storage of multiple bags of smaller volumes is easier than one large holding tank, which is required to be moved through the facility. However, when disposable systems are used, the compatibility to the specific buffers must be determined by evaluating the extractable profile or at least a TOC (Total Organic Carbon) analysis. Data of compatibilities to different buffers can also be obtained from the disposable system vendor. Similarly any filtration device applied to filter the specific buffer must be tested in regard to its compatibility under the process conditions of the specific practice.

Since buffers are commonly of high purity the filter performance criteria focus on flow rate and not total throughput. A premature blocking of the filter is often not experienced. Flow though is the determining factor of process time within the buffer preparation process. The faster the flow rate of the filter the higher the equipment utilization. The better the flow rate of the filter the lower the required EFA, and therefore, the cost per liter will be reduced. For example, a low flow rate (2500 L/h), 0.2 μm-rated filter would require 48 min to filter a 2000-L volume versus only 20 min for a high flow

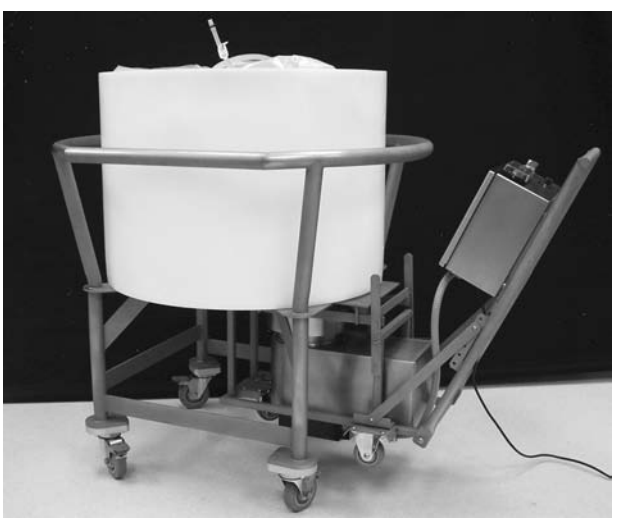


FIGURE 11 Disposable, levitating mixing system.

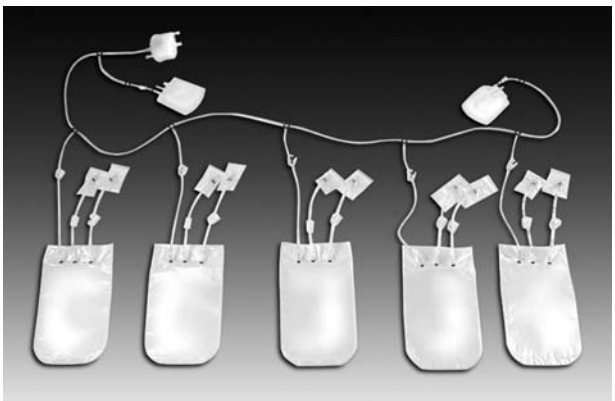


FIGURE 12 Disposable holding bag manifold.

filter (6000 L/h). This would reduce equipment use time by half or the EFA could be reduced, which would cut filter costs.

Another important factor to consider is the buffer's pH range or the variety of buffers used. In certain pharmaceutical processes the pH ranges from 1 to 14, the full spectrum, which some polymers are capable of withstanding and others are not. Again filter vendors are aware of this fact and have developed high flow filters often with a polyethersulfone base polymer as this material is compatible over the entire pH range.

Filter Designs

As mentioned above, flow rate is the key performance parameter in buffer filtration. However, to gain optimal flow rates from sterilizing grade membrane filters, there are limited parameters which can be controlled within the filtration process. Either the differential pressure can be raised, or larger filter surface can be applied, which raises the consumable and capital investments costs. In instances of higher viscosity buffers, the temperature could be raised to lower the viscosity. Every centipoise of viscosity increase lowers the flow rate accordingly.

Since the process conditions are restrictive and might not be able to achieve optimal performance, specific high-flow membrane filter designs have been developed by the filter vendors. Such filters are optimized in respect to the design of the filter membrane and the construction of the filter.

Flow rate increases due to membrane designs are commonly achieved by high porosity, low thickness, and high pleatability. The higher the porosity, the thinner the membrane, and the lower the flow resistances through the membrane in accordance to Darcy's law, in which permeability of the porous media is directly proportional to the fluid flow rate, and membrane thickness is inverse proportional. However, thickness of a membrane has also an effect of long-term retentivity assurance and therefore a careful balance has to be observed (Pall et al., 1980). To lower flow resistance, many of the high flow filter devices use asymmetric membrane structures that seem to channel the fluid stream better through the membrane structure. Asymmetric membranes also include the additional benefit of fractionate retention and therefore distribution of the contaminant.

To achieve a larger EFA within the confinements of the filter cartridge construction, the membrane must be easy pleatable. If the pleatability of the membrane is not a given, the EFA of such a filter is commonly low and/or the pleat edges are too weak to withstand pressure pulsation (Fig. 13). Inappropriate membrane thickness or

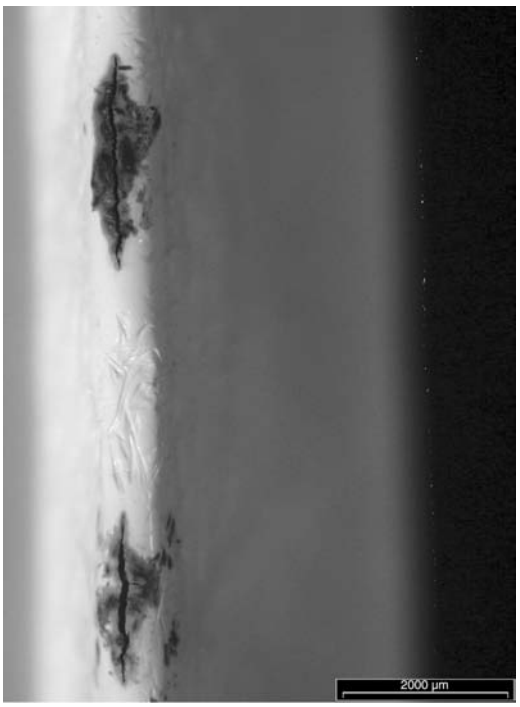


FIGURE 13 Membrane breaks at the pleat edges due to insufficient membrane design and pulsations.

design can create a stretching of the pleat tips. This results in a weakness within the stretched membrane material and physical stress, like steaming or pressure pulsations, can break the pleat tip open. Since both stress factors happen during filtration, this type of filter element design might be undesirable as it could fail integrity after filtration.

Pressure pulsations, caused by water hammer, are not unusual in high flow applications. Any filling processes which utilize rapid valve action and therefore fluid flow and stoppage, might create excessive pressure pulses. The filter cartridge designs are

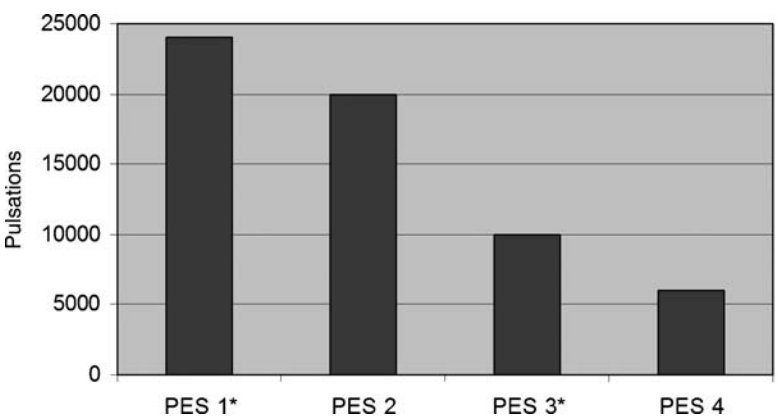


FIGURE 14 Pulsation resistance of different high flow filter cartridge at 5 bar differential pressure (*, double layer filter).

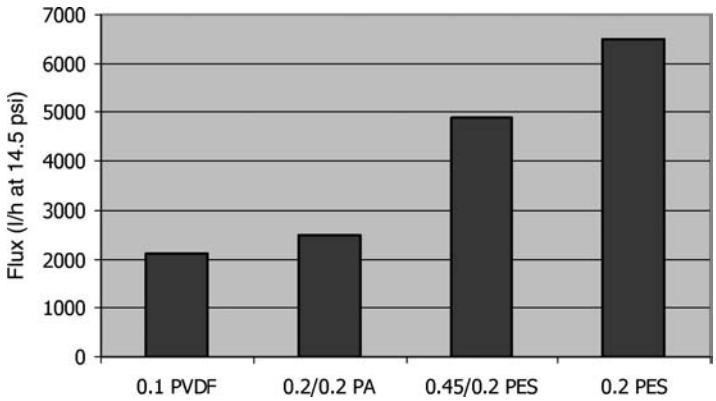


FIGURE 15 Flow rate comparison of different 10-inch filter cartridge configurations.

required to withstand such pulsations and are commonly tested within the vendor's development facilities before released into the market (Fig. 14).

Besides membrane design, filter cartridge design is a key element to achieve optimal flow rate conditions. For example, a single-layer membrane construction will achieve higher flow rates than a membrane double-layer combination, especially when such is of a homogenous (e.g., 0.2/0.2 μm) construct. The flow restriction of a homogenous double layer design can be so high that a single layer membrane filter of a smaller pore size, 0.1 μm rated, might reach a similar flow rate as the 0.2/0.2 μm rating (Fig. 15).

Additionally, the support fleeces, which are layered on top and bottom of the membrane, are required to be of appropriate structure and thickness. If the structure (for example, the fiber diameter) is too large, the fleece structure could press into the membrane at the pleat tips and cause friction. Fleece with large fiber structures are also difficult to pleat. When the support fleece is too thick, the pleat density will be lower and therefore the EFA will be lower. Conversely, when the fleece layer is too thin the pleat density might be too tight, and uneven flow dynamics happen and only the outer areas of the filter membrane will be used. Again such structural designs have to be well balanced and will take years of testing to optimize. Vendors run a multitude of different performance trials before any design component is defined for a specific filter. Only such trial basis will determine an optimal filter choice for a specific application.

Testing and Sizing Filters

As vendors perform multiple tests within their development process, the filter user requires testing of flow optimized filters of multiple vendors (*i*) to find the best possible filter for the particular application and (*ii*) to qualify a second supply option.

Flow rate depends on the entire filter cartridge design and not solely on the membrane's porosity, thickness, and construction. If a membrane with an exceptional flow rate cannot be pleated it is useless within a filter cartridge construction. The optimization of filtration processes requires tests using comparable filter elements, commonly 10-inch filter cartridges. A side-by-side trial can be performed using only comparable filter elements, as such a test would take into account the entire design of the filter and the membrane design, as well as the EFA, flow distribution due to pleat densities, and the fleece thickness. The test would be performed under the required or

specified process conditions, that is, commonly using a set inlet pressure, while the time to filter the fixed fluid volume will be measured. Such a test set-up can be utilized for different filter types to be tested. Important, however, is that the process parameters are constant. For example the same buffer composition, pressure, and temperature settings must be the same to have a comparable result in order to determine the optimal filter type or combination.

47-mm tests for flow rate can be performed as an indicator when the buffers have certain viscosity differences; however, often the results are of no use when utilized as a process scale indication, as such tests evaluate only the porosity and thickness of the membrane itself. Nevertheless, critical and beneficial parameters of the true filter element, which will be used within the process, are not evaluated. Side-by-side trials employing 47-mm discs cannot determine the true flow rate performance of the filter within the production process (Fig. 16). The chart depicts the true flow rate measurements using 10" filter elements and tests performed with 47-mm discs. The tests have been performed at a set differential pressure of 0.5 bar with water at 20°C. The flow rate results of the 10-inch elements and 47-mm discs were calculated to $L/m^2/h$ to compare the flow rates of the disc and the true flow of process elements.

As Figure 16 shows, the 47-mm flow rate results differ greatly from the 10-inch element flows; that is, 47-mm test discs are inappropriate for use in determining an appropriate filter type and scale. For example, the flow rate of 47-mm disc composite A could have a 20% higher flow rate than the 47-mm composite B. However the 10-inch filter element of composite A has $0.5\,m^2$ filtration area and the composite B $0.7\,m^2$ filtration area, which represents a 40% higher filtration area and a respectively higher flow. The 47-mm flow rate test would have misguided the user to the incorrect filter composites and perhaps process filter device, which seems to have a higher flow in the small scale test, but truly has potentially a 20% lower flow rate. Comparisons of flow rate of different filters should not be performed with 47-mm discs as such tests just mislead

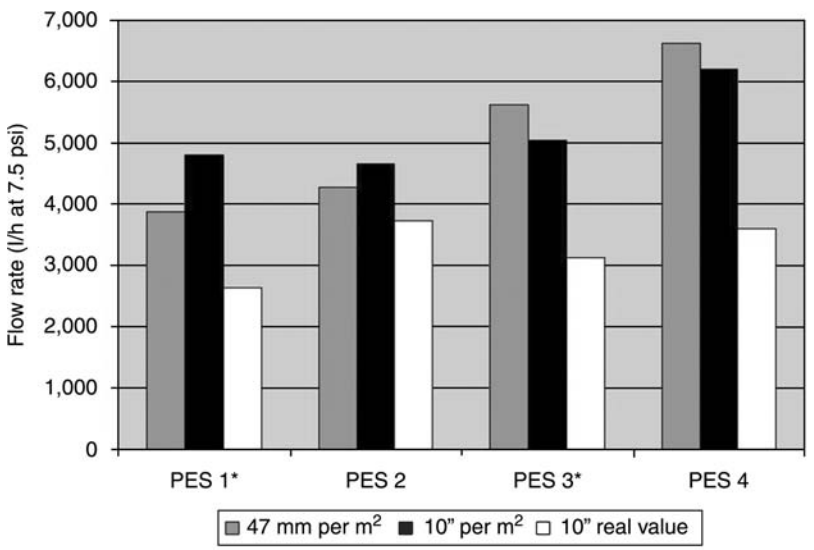


FIGURE 16 Flow rate comparisons of 47-mm discs and 10-inch elements (extrapolated to $1\,m^2$) and actual 10-inch cartridge flow (*, double-layer filter).

the end user. Such tests are only time consuming and are not of true value. Only large scale trials can determine the best flow rate filter.

A benefit of 47-mm test composites is the possibility to check chemical compatibility with lower buffer volumes required. The chemical compatibility needs to be tested as some of the membrane polymers do not fit to certain buffer pH ranges. The 47-mm disc could be flushed with the buffer in a recirculation mode under the future process conditions to find out whether any subtle incompatibility exists. An integrity test, TOC or pH/conductivity test before and after filter contact can create an indication whether the filter polymer is the right choice or not.

High flow rate filters often find the restriction in flow also within the adapter or inner core design of a filter. The smaller the inner core or adapter diameter, the higher the resistivity to flow. Small scale device trials would not be able to determine such influence. Only full scale trials will reveal any potential slow-down of flow due to insufficient adapter or pipe diameters.

Another benefit given by large scale trials with the actual filter device is the possibility of appropriate sizing. Even when safety factors could be used with 47-mm test devices, often the system would be oversized. Some users might consider this as a cost of business; however the costs of such casually sized system could be twice as high as an accurately sized system. The costs are not only attached to the price of such filter devices, but also the hardware required, flush volumes, sterilization times, set-up times and cost of waste. The entire process must be evaluated before an inaccurate decision is made.

CONCLUSION

Years of use and testing have shown that there is no such thing as the perfect filter device, an overall encompassing filter which will serve every application best. The variety of applications and process settings require filters that are not developed to be the best in every performance aspect, but rather be the optimal in a specific performance criteria looked for in a specific application. This chapter touched on two very specific applications that require a completely different scope of filter design and testing. It shows that in the future, filter devices, as well as any other equipment used within biopharmaceutical processes, will be developed according to application needs instead of product features. The one-filter-fits-all approach is as obsolete as the one pharmaceutical process fits all. To find the optimal filter, filter users and vendors have to work together in a very close cooperation to achieve the goal, reduce costs, maintain the yield, and meet the process requirements. Costs are not saved by discounts, but by optimizing the process step within the application and all parameters surrounding this step, including waste removal.

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18
Downstream Processing

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INTRODUCTION

Overview of Downstream Processing in the Biopharmaceutical Industry

Biopharmaceutical manufacture is conventionally divided into upstream and downstream phases, the former dealing with the growth of biological raw material and the latter with the extraction and purification of the desired product. Downstream processing can thus be defined as the series operations that takes the output from upstream processing (e.g., fermentation broth, plant tissue, animal tissue, milk, serum) and yields a stable, pure product (Kalyanpur, 2000). Many different product types can be considered under the umbrella of biopharmaceuticals, ranging from small molecules (secondary metabolites, amino acids etc) to proteins, nucleic acids, viral particles and even whole cells. This chapter focuses on the production of biopharmaceutical proteins (Fig. 1).

Downstream processing is itself usually divided into two main steps: isolation and purification. The isolation step follows immediately after harvesting of the raw material. In the isolation step, the crude raw material is refined into a clarified feed stream, that is, a process intermediate that is free from cells and other particulate matter. The unit operations employed in the isolation step depend very much on the initial raw material. Some biological materials are already clarified to a degree, and require only limited centrifugation or filtration prior to the purification step. Examples include serum, urine and the extracellular medium from the culture of microbes or mammalian cells. A popular strategy for cell culture-based production is to express the recombinant protein as a secreted product, allowing it to be collected from the medium, which tends to contain few contaminants, certainly compared to homogenized cells (Birch and Racher, 2006). In other cases, the raw material can be extremely complex, and isolation may involve multiple operations to remove particulates, fines and other contaminants. This often is the case for intracellular products, where the raw material might be homogenized cells, shredded leaves or ground seeds, and complex body fluids such as milk (Nikolov and Woodard, 2004). It may be necessary to add water to certain raw materials in order to facilitate protein extraction. This water is also considered as a contaminant and the isolation step should therefore achieve volume reduction as well as clarification of the feed stream. A number of different methods may be employed during the isolation step including filtration, gravity separation, centrifugation, flocculation, evaporation,

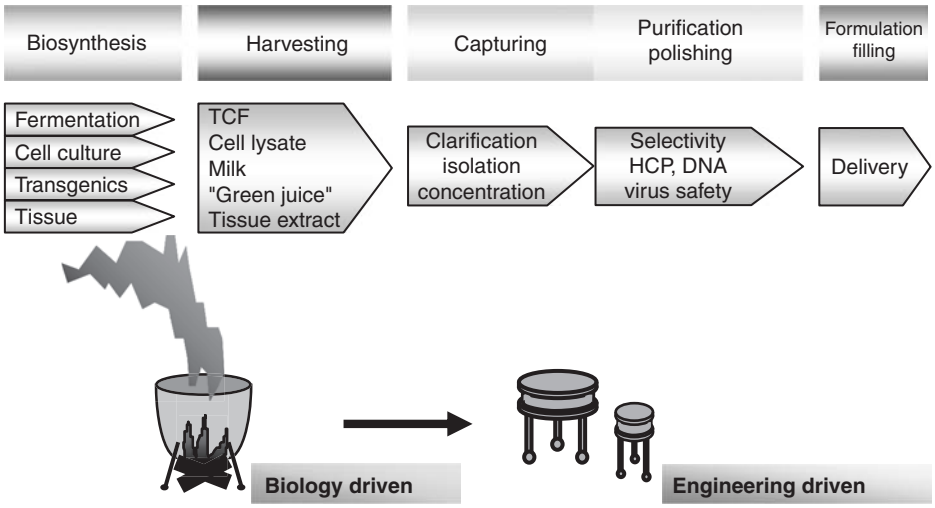


FIGURE 1 A genetic process train for protein pharmaceutical production. *Abbreviations*: HCP, host cell proteins; TCF, tissue culture fluid.

precipitation and extraction. For certain products, affinity chromatography is a key isolation technology, facilitating product capture from the raw material (see below).

In the purification phase, the aim is to prepare a pure biopharmaceutical product from the clarified feed. This is the most challenging and expensive step in downstream processing because it is necessary to separate the desired product from other molecules with similar properties (Levine, 2002; Werner, 2004; Gottschalk, 2005a). High-resolution orthogonal separations are required, and multiple filtration and chromatography operations are usually employed (Somerfeld and Strube, 2005). However, more traditional techniques such as extraction and crystallization could also be used economically in the final stages of purification (polishing) (Gottschalk, 2003).

This chapter considers the principles and applications of different techniques that can be used for the downstream processing of proteins, and explores some of the challenges that remain in this important field.

Challenges in Protein Purification

Some of the major issues which affect protein purification include product loss, denaturation, proteolysis and contamination with other biological molecules, especially nucleic acids, carbohydrates and lipids (Ersson et al., 1989). In small scale preparations, these problems are addressed through the use of an extraction buffer whose pH and ionic strength favor the solubilization of the target protein while other molecules precipitate, and through the addition of detergents, chaotropic agents, reducing agents, proteolytic inhibitors and other additives to protect the target product (Fish, 2004). In large scale industrial processes this is too expensive, and the additives themselves would only need to be removed at a later stage making additional purification steps necessary. Therefore, instead of adding components to protect the target protein, the aim in large-scale processing is to minimize the target protein's contact with potentially damaging agents and remove these agents as early as possible in the process train. Some positive measures can be implemented even in the upstream phase—for example, by ensuring a protein is secreted rather than retained in the cell, it can be recovered directly from the culture

medium without lysing the cells and risking the release of oxidizing agents, proteases and other undesirable molecules. Where an intracellular product is unavoidable (e.g., inclusion bodies in bacteria, proteins expressed in plant leaves or seeds), there is usually a compromise between recovery and purity.

Typically, a target protein will constitute 0.1–5% of the total soluble protein in the raw material, and after the isolation step it will be present as a mixture of many different proteins with a range of physicochemical properties, some very different from the target and some very similar (Doonan, 2000). Purification exploits the unique chemical and physical properties of the target protein in order to obtain a pure and homogeneous preparation.

Important properties of proteins which are exploited for separation include the following:

- solubility,
- charge,
- hydrophobicity,
- size,
- specific binding affinity,
- presence of carbohydrate groups.

Solubility differences are often exploited at an early stage in processing to separate proteins from molecules with very different physical and chemical properties, such as carbohydrates and nucleic acids (Doonan, 2000). Individual proteins also show differing degrees of solubility in a salt solution due to particular surface properties (e.g., charge distribution, juxtaposition of polar and non-polar surface patches etc). This means it is possible to select conditions where a desired protein remains in solution while others precipitate. In a complex mixture of proteins, the solubilities of individual proteins will overlap considerably, so salt fractionation (typically achieved by adding ammonium sulfate to the clarified feed) provides only a crude separation method. However, since ammonium sulfate is inexpensive, salt fractionation can be used economically on a large scale which makes it potentially attractive in biopharmaceutical manufacture. Occasionally, it may also be possible to achieve fractional precipitation with an organic solvent (e.g., ethanol, acetone) although this can lead to protein denaturation at high solvent concentrations. Differential solubilities can also be exploited for separation using two immiscible solvents (see description of liquid/liquid extractions below).

The overall charge of a protein depends on the number of charged amino acid residues on its surface, so all proteins carry a net charge at all pH values except the unique pH (the isoelectric point, pI value) at which the positive and negative charges cancel each other out. Moreover, any proteins that have the same charge at a particular pH may differ in charge at some other pH. A number of methods have been developed to separate proteins on the basis of charge including isoelectric focusing, chromatofocusing, electrophoresis and ion exchange chromatography. While all these methods can be used for small scale preparative purification, only ion exchange chromatography has the combination of simplicity and economy to allow its use in industrial scale processes (Jungbauer, 1993; Desai et al., 2000).

The hydrophobicity of a protein is its tendency to favor non-polar solvents. Like its charge, a protein's hydrophobicity depends on the types of amino acid residues clustered on the surface. Although the surfaces of most soluble proteins are predominantly polar, many of them have patches of hydrophobic amino acids that, under appropriate conditions (usually at high salt concentrations), can bind to hydrophobic matrices. This provides a method for separation in which proteins are dissolved in an ionic solution,

passed over a hydrophobic matrix to which they bind preferentially, and eluted using an organic modifier. Methods based on this property include hydrophobic interaction chromatography (HIC) and reversed-phase chromatography (RPC), although only the former is used for the large-scale purification of proteins since RPC uses strongly denaturing solvents (RPC may be useful, however, for the purification of small peptides).

Proteins also differ from one another in size and shape. This can be exploited in size-exclusion chromatography, where the overall size of a protein determines the likelihood of it becoming trapped in a porous matrix, and in ultrafiltration methods (which utilize very fine filter pore structures and are commonly rated in molecular weight cut-off).

Whereas the methods above depend on differences in protein structure there is also a set of procedures that depend essentially on differences in biological activity (Scouten, 1981; Hermanson et al., 1992; Jones, 2000). In the vast majority of cases, the biological activity of a protein depends on it recognizing and binding to a ligand. For example, enzymes bind to substrates and inhibitors, hormones bind to receptors, antibodies bind to antigens and so on. This specific biological activity can be exploited by the construction of an inert substrate, such as agarose, to which the appropriate ligand is (usually covalently) attached. Passage of a protein mixture through the resulting affinity matrix should result in the binding of one or a small number of proteins that recognize the ligand. Subsequent elution can be achieved by passing a solution of the ligand, or a suitable analogue, through the column, or by using an elution buffer that disrupts the protein-ligand interaction. Methods based on this principle include immunoaffinity chromatography, protein A chromatography for the purification of antibodies, immobilized metal ion affinity chromatography for the purification of proteins with chelating activity, and dye-binding chromatography for proteins that recognize particular organic groups. Lectin chromatography is a specialized derivative which exploits the affinity of lectins (carbohydrate-binding proteins) to bind selectively to specific oligosaccharide groups on glycoproteins. Elution can be effected by passage of a solution of the appropriate monosaccharide through the column.

METHODS USED IN PRODUCT ISOLATION – LOW TECHNOLOGY TECHNIQUES

In contemporary downstream processing, isolation often consists of capturing the product from the clarified feed, leading to a rapid and steep increase in purity and concentration early in the process. Trends include the processing of large tissue culture fluid (TCF) volumes, a high dynamic capacity, high linear flow rates and low contact times. Depending on the efficiency of capture, the purification step may involve some intermediate processing before polishing, or (if the output from the capture stage is already highly pure) two consecutive and orthogonal polishing steps (Gottschalk, 2006a).

Harvesting and capture often consist of a combination of filtration to remove particulates and affinity chromatography to select the target on the basis of its binding affinity, thus removing 99% of contaminants and achieving volume reduction in a single operation (Huse et al., 2002). The use of affinity chromatography at an early stage is advantageous because the desired product is rapidly separated from potentially damaging contaminants such as proteases and oxidizing agents. However, it is not always possible to develop ligands to trap the desired product, and in many cases the cost of affinity media becomes unsupportable as the scale of production increases. Therefore, before considering filtration and affinity chromatography in detail we first

explore some traditional separation techniques which are increasingly used in the preparation of small molecule drugs and could soon be adopted on a more routine basis in biopharmaceutical manufacture.

Gravity Separation and Centrifugation

A particle suspended in a liquid medium of lesser density tends to sediment downward due to the force of gravity. Its passage downwards is opposed by a buoyancy force equivalent to the weight of the displaced liquid, friction between particles and the liquid, and to certain extent diffusion. Particles whose buoyancy force exceeds the gravitational force acting downwards will tend to rise to the surface and float. Gravity separation can therefore be used to sediment heavy particles and float buoyant particles, leaving a partially clarified liquid layer. Conventional gravity separation can be achieved either in a still tank or a stirred vessel. It is rapid and efficient for large particles but slow and inefficient as the particles get smaller, and is therefore only suitable for crude separation steps. More efficient gravity separation can be achieved using a plate separator, which comprises a set of evenly spaced plates usually inclined at an angle to provide a greater settling area in a smaller space. This allows a greater feed flow rate and minimizes the bottleneck during clarification.

Centrifugation is a mechanical process that utilizes an applied centrifugal force in place of gravity to separate the components of a mixture according to density and/or particle size. It is a well-established unit operation whose applications include cell separation from broths, removal of cell debris, separation of protein precipitates, and even the separation of dissolved macromolecules (ultracentrifugation) although this is rare in large scale processes (Taulbee and Maroto-Valer, 2000). Centrifugation is typically a batch operation at the laboratory scale (the feed is placed in a disposable tube or bottle, and the supernatant is recovered from the bottle at the conclusion of the run). However, the typical laboratory centrifuge has a small volume that is insufficient for a process-scale operation, even when scaled up. Therefore, large scale operations tend to use continuous centrifuges, where the feed is continuously fed to a spinning rotor and the supernatant and waste pellet are continuously discharged.

Several different forms of centrifugation have been developed and each has a different application in downstream processing (Fig. 2). *Differential sedimentation* is a form of centrifugation in which the media are within the centrifuge tube or bottle is initially homogeneous. Larger and/or denser particles sediment more rapidly in the centrifugal field and thus form a pellet on the wall or floor of the rotor faster than smaller or lighter particles, which tend to remain in the supernatant. The magnitude of the applied centrifugal force and the duration of centrifugation can be used to determine the size or density of particles that are sedimented. This approach works well when the objective is to pellet solid particles, such as cells or tissue debris, or to clarify a liquid feed stream.

Density gradient centrifugation is similar to differential sedimentation in that the principle is to exploit centrifugal force to separate feed components on the basis of particle size or density. However, the distinguishing feature is that the media are in the centrifuge tube is heterogeneous, and the density of the solution increases along the axis of rotation. This is usually achieved by varying the concentration of solute along the centrifuge tube prior to sample loading. As well as pelleting very dense particles, density gradient centrifugation also achieves the separation of particles within the density range of the solution into different bands. In *rate zonal centrifugation*, all particles will eventually form a pellet so the run must be interrupted to isolate particles in the desired density range. In *isopycnic centrifugation*, a very dense solution is used and particles or

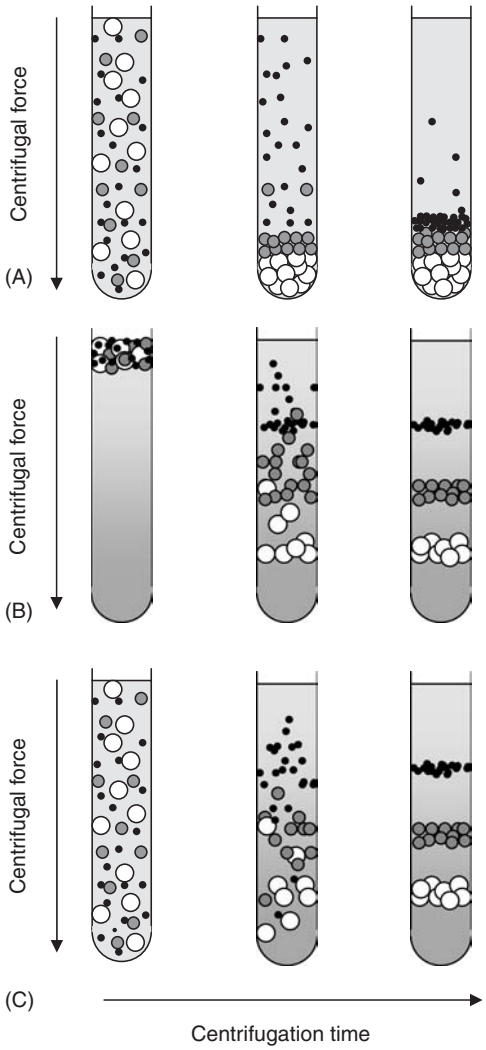


FIGURE 2 Principles of different forms of centrifugation. (**A**) Sedimentation—particles distributed uniformly in a homogeneous solution sediment according to size with the largest particles settling onto the surface of the centrifuge tube first. (**B**) Density gradient centrifugation—particles loaded in a thin zone above a layered solution with increasing density become distributed into density zones as long as centrifugation is not run to completion. (**C**) Isopycnic ultracentrifugation—particles or solute molecules distributed uniformly in a homogeneous solution will form density zones at very high centrifugation speeds as the solute itself forms a density gradient.

molecules in the density range will never form a pellet but will remain suspended at a specific distance along the density gradient. Such processes are common in laboratory scale preparations but are rarely used on an industrial scale.

Several types of continuous centrifuges are incorporated into large-scale processes, and they usually have the ability to separate particles in the 0.1–200 μm diameter range (Zydney and Kuriyel, 2000). A typical example is the decanter centrifuge shown in Figure 3A. The depicted centrifuge is of the 'bowl and scroll' design, in which a rotating

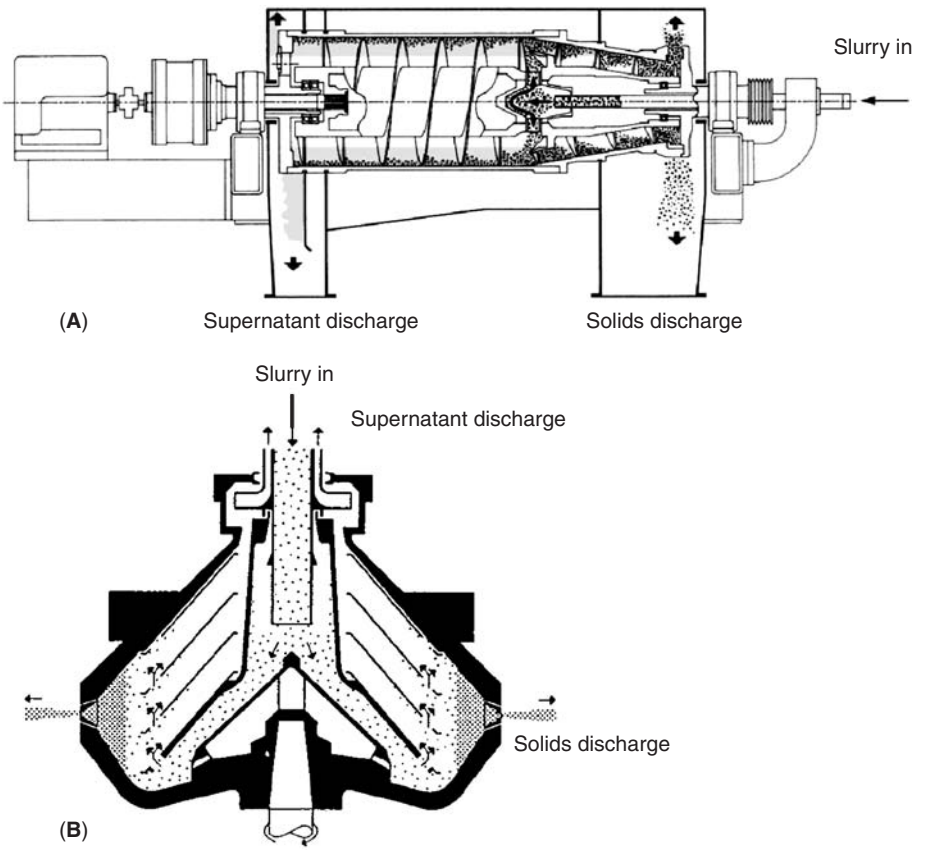


FIGURE 3 Examples of typical continuous centrifuges used in downstream processing. (**A**) A scroll and roll decanter centrifuge. (**B**) A disc stack centrifuge.

scroll (like a screw thread) fits tightly into a conical-shaped bowl whose apex lies at the source of the centrifugal force. The feed is introduced into the middle of the scroll. As the centrifuge bowl rotates, the particles in the slurry sediment upon the walls of the bowl. The accelerating force, which can be up to $10{,}000 \times g$, is optimized for the application and the expected composition of the solid component of the slurry. As the centrifuge spins, the scroll is rotated, scraping the solids towards the apex of the bowl where they are removed. At the same time, the clarified supernatant liquid is directed to a collection device either by gravity discharge or with discharge under pressure. This type of device is best suited to feeds with high solids content, such as fermentation broths or milled plant tissue.

Another continuous centrifuge device often used in large-scale processing is the disc stack centrifuge (Fig. 3B) (Kempken et al., 1995). The disc stack centrifuge uses higher centrifugal forces than the decanter centrifuge, and is therefore suitable for slurries with a lower solids concentration and/or smaller particle sizes. The design incorporates sets of stainless steel plates (the disc stack) which provide a greater sedimentation area and significantly accelerate the separation process. The solids deposited on the plates can be removed continuously, intermittently (semi-batch mode) or regularly (batch mode) depending on the application. A typical application for semi-batch or batch mode centrifugation is the removal of mammalian cells after batch or fed-batch fermentation. Centrifuges are effective in reducing cell concentrations and larger debris particles, but

they tend to perform poorly in removing small cell particles and colloidal compounds which are always present in biopharmaceutical feed streams.

In laboratory scale operations, centrifugation is often combined with filtration, such that the feed stream is centrifuged through some form of filter matrix to trap particles above a certain size and prevent them sedimenting onto the walls of the tube. Similar to conventional filtration achieved via differential pressure across a membrane or pad (see below), centrifugal filtration is driven by the pressure exerted by a liquid medium within a centrifugal field. Opposing the centrifugal pressure is the combined resistance of the porous medium and filter cake. Although spin-filters are too complex and expensive to scale up for very large-scale processes, separators are often linked to depth filtration with lenticular filters, so that particles and debris remaining after separation are removed (see filtration section below).

Extraction and Precipitation

Extraction is the process of moving one or more components of a mixture from one phase to another, while precipitation is the process of removing one or more components from solution to form a solid phase (the precipitate). These are among the simplest and least expensive fractionation methods since they can be achieved by the addition or removal of salt, organic solvents or by changes in temperature and pH.

Liquid-liquid extraction using organic and aqueous extraction media is a traditional separation operation that can be applied to the purification of biopharmaceuticals. In three-phase partitioning, proteins can be purified directly from cell homogenates by partitioning between a layer of butanol and a strong aqueous salt solution. Under these conditions, cell debris tends to separate into the organic phase and nucleic acids precipitate at the interphase while proteins remain in solution. Several proteins have been purified using this method (Paule et al., 2004) while the selectivity of extraction can be increased through the inclusion of affinity reagents such as metal ions in the system (Roy and Gupta, 2002).

Aqueous two-phase extraction (ATPE) systems are the most widely used extraction operations, employing a mixture of aqueous polymers and/or salts (Kula and Selber, 1999). One phase generally contains polyethylene glycol (PEG) and the other contains a different polymer, such as dextran, or the salt potassium phosphate. Under ideal conditions, the desired protein can be separated into the PEG phase while the majority of contaminating proteins as well as other contaminants are trapped in the second phase, or in the interphase, and can be removed by centrifugation. Recent developments in aqueous two-phase extraction include phase-forming systems containing reagents that are less expensive and easier to process than the traditional reagents (Li and Peeples, 2004). Advances in the hardware used with ATPE systems have been reviewed by Banik et al. (2003).

Protein precipitation is a well-established but poorly understood process. Under mild conditions, protein precipitation is reversible and subsequent redissolution restores total activity. Protein precipitation may be either a product isolation step or a purification step. In the former case, precipitation and subsequent redissolution in a smaller volume of water not only reduce processing volume, but the resulting solution also contains mostly dissolved protein, free of other soluble contaminants. In the latter case, the differential solubility of proteins may be used for fractionation. Proteins can be precipitated by changing the pH or temperature or by adding a mild organic solvent, a salt, a multivalent metal ion, or a nonionic polymer. The chosen precipitation method depends on whether the protein precipitate that is formed can be re-dissolved without loss of activity, the expense of the precipitating agent and its recovery, and the effects of precipitating agent

impurities in the precipitate. The following methods are widely used (Cohn and Edsall, 1943; Kumar et al., 2003; Hilbrig and Freitag, 2003).

1. *Salting out*. High salt concentration promotes protein aggregation and precipitation. Although the mechanism is not well understood, the salt is thought to remove the water of solution from the protein, thereby reducing protein solubility. The Hofmeister series represents decreasing anion effectiveness: citrate > phosphate > sulfate > acetate > chloride > nitrate > thiocyanate. The salts at the low end of this series cause structural damage to proteins. The high solubility of ammonium sulfate in water and the position of sulfate in the Hofmeister series make it the most popular choice for salting out proteins.
2. *Metal ions*. Ions such as Mn^{2+}, Fe^{2+}, Ca^{2+}, Mg^{2+} and Ag^{+} bind to different protein functional groups and can cause them to precipitate. They act at much lower concentrations than the ions of the Hofmeister series and are easily removed by ion-exchange adsorption or chelating agents.
3. *Adjusting the pH*. Proteins are soluble in water due to the interaction of their charged groups with ionized water molecules. Adjustment of the pH to the isoelectric point (pI) yields minimum solubility, since the net charge of the protein is eliminated. Most proteins have a pI < 7, and the relatively low cost of acids makes pH adjustment with acid a popular method of protein precipitation. However, too much acid or base can cause irreversible denaturation.
4. *Organic solvents*. Addition of a mild organic solvent to an aqueous protein solution reduces the solvent dielectric constant, thereby inducing protein precipitation. The solvent must be completely miscible with water (e.g., ethanol and acetone). Solvent precipitation is typically performed at low temperature (< 10°C) because conformational rigidity then prevents irreversible denaturation.
5. *Temperature*. Proteins precipitate and denature at different rates when the temperature is increased. However, some robust proteins are resistant to heat denaturation. Therefore, by subjecting an impure mixture to an elevated temperature for an appropriate period of time, a protein can be purified in solution due to the irreversible denaturation and precipitation of the impurities.
6. *Polymers and polyelectrolytes*. Nonionic, water-soluble polymers induce protein precipitation by excluding water from the solvation structure of a protein. PEG is the most widely studied and widely used polymer, but dextrans are also used for this purpose. The effect of polymers as precipitating agents is similar to partitioning in aqueous two-phase polymer systems (see above). High PEG concentrations are required to precipitate low-molecular-weight proteins, whereas low concentrations are required for high-molecular-weight proteins. Polyelectrolytes such as polyacrylic acid, carboxymethyl cellulose and polyethyleneimines precipitate proteins at a much lower concentration (usually < 0.1%) than nonionic polymers. They act more like flocculants and adsorb to the protein. Thus, polyelectrolytes, unlike PEG, coprecipitate with the protein and can cause irreversible denaturation.

Evaporation

In some biomanufacturing processes, evaporation may be a suitable volume reduction step. To minimize protein denaturation, vacuum evaporation may be used. Operating temperatures < 40°C are desirable. Foaming often causes equipment fouling and protein denaturation at the air-water interface. Ultrafiltration (see below) is a much gentler

method of volume reduction for proteins, and has largely replaced evaporation in industrial-scale applications.

Flocculation

Flocculation is a similar process to coagulation, where suspended particles clump together because the attractive forces between them overcome any repulsive forces caused by like surface charges (Shaeiwitz and Henry, 2005). Such repulsive forces can be eliminated, for example, through the addition of inorganic electrolytes, which shield the surface charges, or by the addition of polyelectrolytes that bind to and neutralize the surface charge. Flocculation is the agglomeration of particles due to the bridging effect exerted by polymers that are adsorbed to more than one particle. Coagulation and flocculation probably occur simultaneously when polymeric polyelectrolytes are added. Flocculation has been used mainly for the removal of whole cells from fermentation broth, although in principle the technique could also be applied to cell debris and proteins. Particles are often coagulated or flocculated prior to filtration to reduce the passage of small noncoagulated particles through the filter and to produce a more porous cake which is easier to remove.

FILTRATION METHODS USED IN PRODUCT ISOLATION AND PURIFICATION

Overview

In downstream processing, filtration refers to any process in which a liquid feedstock containing suspended particles or dissolved molecules is forced through a selectively permeable medium, such as a microporous membrane, such that only certain components of the feed pass through into the permeate while other components are retained on or in the filter medium (the retentate) (Jornitz and Meltzer, 2004). Filtration predominantly separates the components of a mixture according to particle or molecular size, since the minimum pore size is the most important characteristic of any filtration device. However, more sophisticated matrices have chemically modified surfaces which can specifically trap particular molecules based on their affinity or physicochemical properties, making such separations equivalent in practical terms to corresponding chromatography steps (see below). Filtration is a mechanical process, and a driving force across the filter medium is required. The driving force can be generated by gravity, a pressure differential, the application of a vacuum, a centrifugal force (see centrifugal filtration above), a difference in concentration or electrical charge across the medium, a temperature differential, or in some cases a specific form of chemical attraction or repulsion (Jornitz et al., 2002).

Filtration is applied at many different stages in downstream processing, both during product isolation and purification. Filtration methods are applied widely in biomanufacturing processes because they operate at relatively low temperatures and pressures, and require no phase changes or chemical additives. Thus, these processes cause minimal denaturation, deactivation or degradation of labile macromolecules such as proteins. Filtration methods are often subcategorized on the basis of retentate size, as summarized in Table 1 (Zeman and Zydney, 1996). *Microfiltration* is used throughout the process train, for example, for cell/particulate removal during clarification and for sterile filtration towards the end of the process. When microfiltration is used as a clarification method, the product stream may comprise either the retained cells on the upstream side of

TABLE 1 Different Forms of Filtration Used in Downstream Processing

Method	Pore size	Retained	Applications
Microfiltration	100 nm to 10μ m	Cells, cell debris	Clarification, sterile filtration (fill and finish)
Ultrafiltration	10–100 nm (M_r 10^3–10^6)	Fine particles, viruses, large proteins	Clarification, virus clearance, size fractionation of proteins, concentration, diafiltration
Nanofiltration	1–10 nm ($M_r < 10^3$)	Nucleic acids, viruses, proteins	Purification of proteins, virus clearance
Reverse osmosis	0.1–1 nm ($M_r < 10^3$)	Salts, sugars	Water purification

the filter (containing an intracellular product) or, if the product is secreted, the filtrate on the downstream side (Jornitz et al., 2003). The membrane pore size in microfiltration ranges from 0.04–3.0 μm depending on the specific application.

Ultrafiltration is similar in principle to microfiltration, but the pore sizes are an order of magnitude smaller and are used for the retention of molecules in solution as well as suspended particles (Dosmar and Brose, 1998). For this reason, the retentate can be expressed either in terms of particle size or in terms of molecular weight (Table 1). Ultrafiltration in the lower size range can be used to size fractionate proteins, and separate target proteins from buffer components for buffer exchange, desalting or concentration. Another important application is to ensure virus removal in later processing steps, using filters with pore sizes up to 50 nm. This requirement originated from process development for the purification of plasma proteins, as discussed by Walter et al. (1998) and Sofer (2003) as well as in relevant regulatory documents and technical guidelines (CPMP, 1996; 1997; PDA, 2005). The validation of virus removal by ultrafiltration is discussed by Immelmann et al. (2005).

Ultrafiltration membranes are available in many different materials that provide alternatives in terms of chemical compatibility and tendency to suffer from fouling. Low-protein-binding variants (e.g., cellulose acetate, polyacrylonitrile–polyvinylchloride copolymer, modified polyethersulfone) are designed with biopharmaceutical manufacture in mind. The choice of membrane for different applications is discussed by Rubin and Christy (2002).

Nanofiltration and *reverse osmosis* are similar to ultrafiltration but the pore sizes are even smaller and the molecular weight cut-off points even lower. They are typically used to separate low-molecular-weight molecules from water and other solvents, for example, in desalination or the preparation of pure water. In biopharmaceutical manufacture, such membranes are used for buffer preparation and formulation.

Configuration and Design of Filter Modules

There are two main configurations of filter devices in downstream processing (Fig. 4). In *dead-end filtration* (also known as *normal-flow filtration*) the feed stream is perpendicular to the filter device, which is usually a membrane or pad. The filter device effectively blocks the feed, which must be forced through it under pressure. Because this configuration inevitably leads to the rapid build up of retentate on the feed-side filter surface, it is used mainly for laboratory scale processes if the feed contains a high concentration of the retained species. For large scale processes, dead-end filtration is

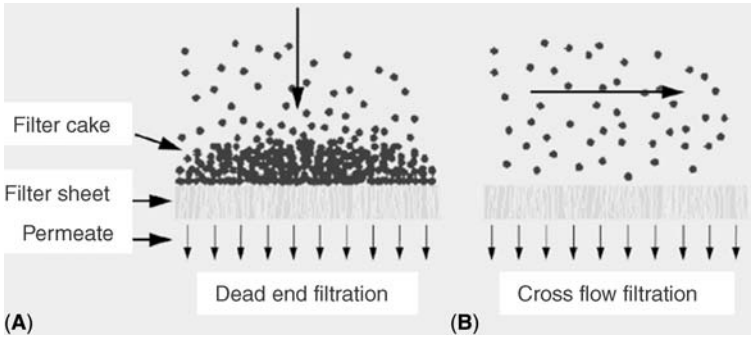


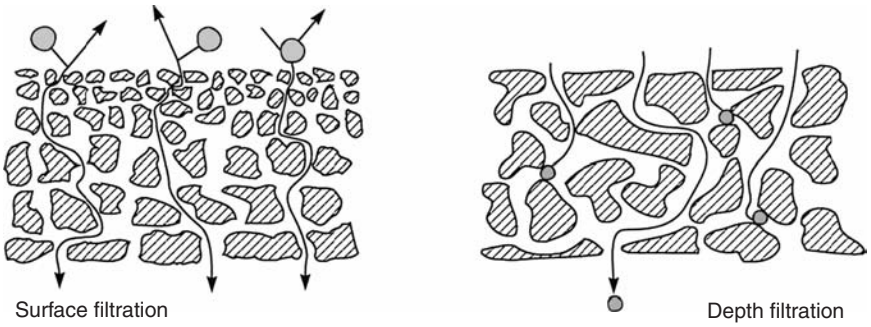
FIGURE 4 Comparison of (**A**) dead-end (normal flow) and (**B**) tangential (cross-flow) filtration. In each panel, the large arrow shows the direction of feed flow and the small arrows show the direction of permeate accumulation.

only used where the retentate load in the feed stream is anticipated to be low (e.g., sterile filtration for product filling, virus removal). Where filtration is used for clarification and size fractionation, *tangential-flow filtration* (also known as *cross-flow filtration*) is preferred. In this configuration, the feed flow is parallel to the filter medium and thus perpendicular to the flow of permeate. This allows retained species to be swept along the filter surface and out of the device, helping to maintain high flux levels even with large amounts of retentate.

Tangential-flow filter modules come in many designs. These differ in terms of channel spacing, packing density, cost, pumping energy requirements, plugging tendency and ease of cleaning, so the design must be chosen on a case-by-case basis for each bioprocess depending on the implications of the above criteria in the context of the overall process train (Jornitz et al., 2002; Dosmar et al., 2005). Filter media can be divided into two major types: surface filters and depth filters (Fig. 5A). *Surface filters* are essentially thin membranes containing capillary-like pores. Particles or molecules which are too big to pass through the pores are retained on the membrane surface, that is, the retentivity is "distinct" at a certain particle-size in regard to the pore size/cut-off and this can be validated as discussed below.

In contrast, *depth filters* have a thicker 'bed' of filter medium rather than a thin membrane, and particles are trapped in the interstices of the internal structure, which describes a torturous path from one side of the filter to the other. To increase the surface area available for filtration without increasing the footprint, depth filter pads are either inserted manually into filter presses or, more preferably, supplied as lenticular filters, in which multiple filter pads are pre-assembled in a modular housing.

The materials used to construct depth filters include cellulose fibers, inorganic filter aids, resin binders and synthetic polymers, offering a large inner surface area and void volumes of up to 85% (Singhvi et al., 1996; Prashad and Tarrach, 2006). Inorganic filter aids such as diatomaceous earth and perlite increase the permeability and retention characteristics of the filter matrix, while synthetic polymers and resin binders increase the strength of the filter medium and generate a net positive charge that helps to trap colloids. For these reasons, depth filters can trap particles much smaller than the maximum pore size. The retention mechanism of depth filters is not absolute, and changes during operation as retentate builds up in the filter matrix. Therefore, depth filters cannot be validated for use in sterile filtration in the same way as membrane filters, but because they are less expensive than membrane filters they are often employed in pre-filter steps to remove cells and debris. For example, feed streams from mammalian cell



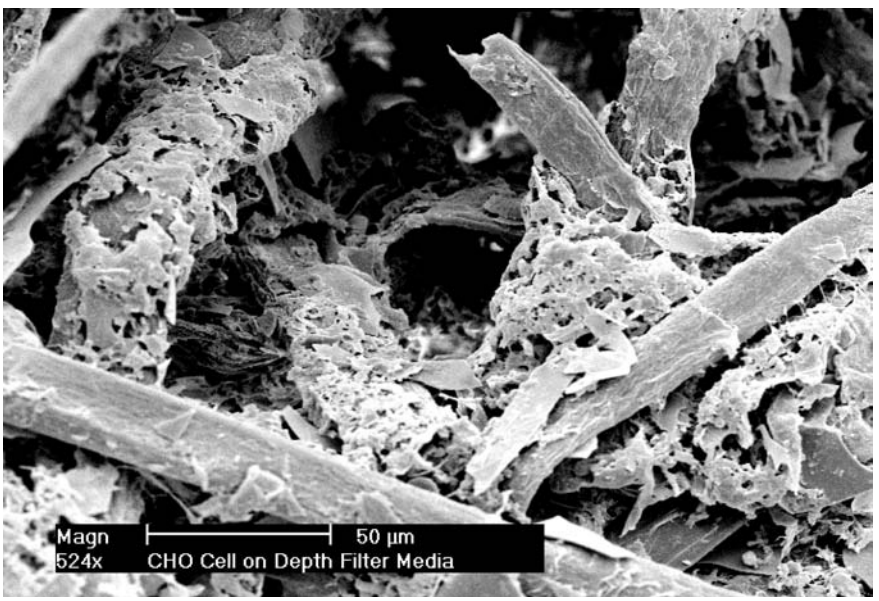


FIGURE 5 Comparison of membrane and depth filters. (**A**) The filtration mechanism of membrane filters (*left panel*) is absolute, with particles above a certain size rejected at the surface and smaller particles allowed through to the permeate, whereas that of depth filters (*right panel*) is not absolute, with particles becoming trapped in the internal matrix but some getting though. (**B**) Micrograph of Sartoclear® P depth filter media that has been used for the clarification of CHO cell broth, showing trapped CHO cells on the cellulose fibers.

cultures often feature two depth filters in series to clarify the fermentation broth for reactor volumes up to approximately 3000 L. For greater volumes, a continuous centrifuge is typically used to remove the largest particles followed by depth filtration and membrane filtration in series to remove smaller particles and fines. A Sartoclear® P depth filter that has been used for the clarification of Chinese hamster ovary (CHO) cell broth is shown as an example in Figure 5B.

Filtration Efficiency

The capacity of a filtration process is usually expressed in terms of *flux*, which is the volume of permeate passing through a particular membrane area per unit time (usually liters per square meter per hour) (Dosmar et al., 2005). The driving force for flux is the

pressure difference between the feed and permeate side, which is known as the transmembrane pressure. Opposing this is the viscosity of the liquid and the hydraulic resistance of the membrane, which depends on the pore size and distribution. The permeability of the membrane is operationally defined as the inverse of its hydraulic resistance.

For a pure water feed, flux increases linearly with transmembrane pressure because resistance and viscosity remain constant. During a typical filtration process, however the flux tends to decline over time because both viscosity and resistance increase. During cross-flow filtration, flux declines rapidly at first, then the decline slows down, and eventually a steady state is achieved. This reflects two simultaneous phenomena – cake layer build up and fouling. *Cake layer build up* occurs in microfiltration when retentate particles accumulate on the feed side and achieve a packing density that causes them to form a defined layer on top of the membrane. This has two consequences. First, the cake layer resists the flow of permeate by effectively acting as an additional filter bed, increasing the thickness of the filter membrane, and leading to increased resistance. Second, the local viscosity of the feed is increases because the concentration of particles near the filter surface is higher than in the bulk feed, resulting in reduced flux. In ultrafiltration, the equivalent phenomenon is known as *concentration polarization*, where the retentate remains in solution but builds up on the feed side, increasing in concentration to such an extent that the osmotic pressure of the retained solute opposes the force driving the permeate across the membrane. At very high concentrations, the retentate can approach its solubility limit and form a gel layer on the membrane surface that also obstructs the flow of permeate (Dosmar and Brose, 1998).

Cake layer build up and concentration polarization are both consequences of the retentate accumulating on the feed side, and both can be reversed by cleaning or back-washing, since they do not permanently affect filter performance. On the other hand, *fouling* is a distinct phenomenon in which molecules in the feed physically and chemically interact with the membrane leading to a permanent loss of function. Examples include the adsorption and deposition of macromolecules, cell fragments or small organic molecules on the membrane surface or within the pores. Fouling increases the hydraulic resistance against permeate flow, and may also increase the observed retention of the membrane as it reduces the effective pore size. As stated above, membranes used in biopharmaceutical manufacture are designed to prevent the binding of proteins as much as possible, thereby reducing fouling and helping to avoid the loss of product through binding to the filter medium.

Validation and Integrity Testing

The suitability and reliability of a membrane filter for particular manufacturing purposes is established through basic performance and integrity tests (Jornitz and Meltzer, 2003). This is important because the selectivity of a filter is determined by its largest pores and any weakness or loss of integrity could lead to contamination of the filtrate with molecules or particles from the retentate, including viruses and prions (CPMP, 1996; 1997; Immelmann et al., 2005). Filters for microfiltration and ultrafiltration are usually tested beyond recommended tolerances using the equipment, physical conditions and material requirements of intended process, according to the example criteria listed below (further details can be found in Chapters 11, 13, and 14 of this volume):

- bubble point testing to establish the largest pore size;
- testing for extractables (particulate or soluble contaminants originating from the filter medium or equipment);

- flux using pure water;
- pore size, established using a range of polymers of known size.

CHROMATOGRAPHY TECHNIQUES FOR PRODUCT CAPTURE AND PURIFICATION

Overview

Chromatography refers to any procedure in which the components of a mixture are separated by distribution between two phases for which they have differing affinities. Among the many different chromatography formats available, the one that is usually applied in large-scale downstream processing is column-based liquid chromatography, in which a liquid feed stream is passed over or through a porous, solid matrix or resin held in a column (Desai et al., 2000; Jagschies, 2006). The components of the mixture become distributed by virtue of their relative affinity for the solid and liquid phases. In most cases this is based on selective adsorption to the resin (i.e., there is some kind of molecular interaction with the resin), although gel filtration (size exclusion chromatography) is an exception as discussed below.

The general procedure for adsorptive chromatography is to introduce the clarified feed stream into the column under conditions where certain components bind strongly to the resin while others flow through. The composition of the buffer is chosen to favor the retention or elution of specific components. By changing the composition of this buffer, molecules that initially bind to the resin can be washed through in subsequent fractions. Chromatography columns can be run in retention mode, where the target protein is retained and impurities are washed through, or in flow-through mode, where impurities are retained and the desired protein is eluted. Affinity chromatography is usually run in retention mode, and the interaction between the target molecule and the resin is so specific that only a few species are retained while most impurities are washed through. Elution usually occurs in a single step to recover the target protein. The other adsorption chromatography methods exploit more general physicochemical properties and both the retained fraction and the eluate are usually complex. For this reason, elution is typically performed with a gradually changing buffer composition to elute a series of fractions whose components have gradually increasing affinity for the resin. In large-scale processes, gradient elution may be replaced with stepwise gradients which are easier to automate, although programmable linear gradients are becoming more common. Adsorptive column chromatography is particularly applicable in downstream processing because short columns with a large diameter (up to 2 m) can achieve very high flow rates of 300–500 cm h^{-1} (Jagschies, 2006). The power of chromatography as a purification strategy is that two or more different operations can be carried out in series to achieve maximum separation by exploiting different principles. In this regard, the logical sequence of chromatographic steps should take into account the composition of the feed and the starting and elution conditions. For example, hydrophobic interaction chromatography generally begins with a high salt buffer but the eluting buffer is of low ionic strength. The converse applies in ion exchange chromatography, so where both are used in a given process it makes sense to place these operations back-to-back.

Affinity Chromatography

In affinity chromatography, the desired product is adsorbed onto a resin containing a covalently-bonded specific ligand, usually but not always reflecting the biological

function of the target molecule (Wilchek and Chaiken, 2000; Chaga, 2001; Turkova, 2002). The ligand exploits the complexity of the target protein's structure, which consists of four basic intermolecular binding forces—electrostatic bonds (salt bridges), hydrogen bonding, hydrophobic forces and van der Waals interactions—distributed spatially in a defined manner. The degree of accessibility and spatial presentation within the resin, and the strength of each force relative to each other, dictate whether these forces are utilized to effect the separation. The overall affinity of the biological recognition between ligand and counter-ligand reflects the sum of the various molecular interactions existing between them. However, various ligands may be found that emulate some or all of the available binding forces to various degrees. Therefore, in some cases, the ligand may be absolutely specific for a particular protein, for example, an immunoglobulin raised against a specific target, or an immobilized substrate which will capture the corresponding enzyme. In other cases the ligand may recognize a particular family of proteins, for example, Protein A will purify any immunoglobulin G (Ey et al., 1978; Suralia, 1982; Huse et al., 2002). General or group-specific ligands can be used to select proteins with particular characteristics, for example, immobilized metal ions can be used to trap proteins with chelating activity, as is the case for His-tagged recombinant proteins (Loetscher et al., 1992; Crowe et al., 1994). There are also large numbers of artificial ligands such as textile dyes and those based on rational design or the screening of chemical libraries (Curling, 2004a,b). Specific interactions can be used to trap suitably labeled proteins, for example, proteins modified to contain particular epitopes or fusion partners can be trapped by corresponding antibodies or binding partners, and proteins labeled with biotin can be trapped by their affinity to avidin.

Because affinity chromatography is highly selective, it is the favored method of product capture (the initial stage of isolation). Unlike the other chromatography methods discussed below, the capacity of the resin is not compromised by the binding of hundreds of foreign proteins, since only those proteins with very specific binding affinity will adsorb to the matrix. This means that purification factors in the tens of thousands can be achieved in principle, although the actual purification factor rarely exceeds 100 in practice. In the biomanufacture of antibodies for example, the isolation stage generally comprises a microfiltration step followed by Protein A affinity chromatography. This results in the elimination of all particulates and 99% of soluble impurities in the first two steps (Huse et al., 2002). The eluate from the affinity chromatography column can then be applied directly to downstream chromatography steps to separate the antibody from degradation products and column extractables, such as leached Protein A.

In a typical process (Fig. 6), the column is first equilibrated with a buffer reflecting the optimum binding conditions between ligand and counter-ligand. Clarified feed is introduced into the top of the column and allowed to percolate through the resin under gravity, or with positive pressure. The feed may also be adjusted, in terms of ionic strength and pH, to optimize binding. The column is then washed with several volumes of the equilibration buffer to wash through unbound components. This step has been completed correctly when the UV absorbance of the eluate returns to the base line level.

Recovery of the bound protein is achieved by desorption, which in this case means the disruption of ligand/counter-ligand binding by changing the binding equilibrium. Depending on the ligand, this can be achieved specifically or non-specifically. Specific desorption relies on competition for ligand binding sites between the adsorbed protein and a free counter-ligand which is added to the elution buffer at the appropriate concentration. An example of specific desorption is the elution of bound glycoproteins from a lectin affinity column, which is achieved through the addition of competing carbohydrates. Advantages of specific desorption include the requirement for only low

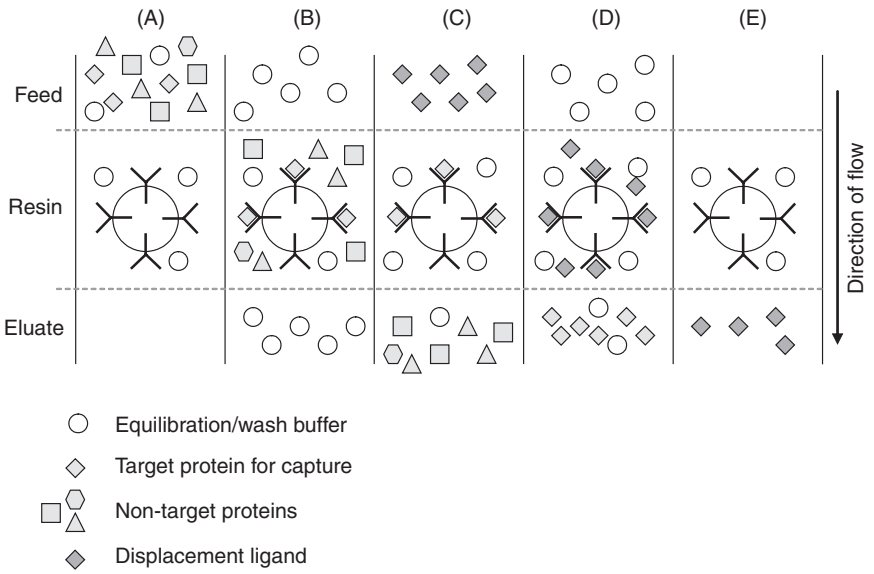


FIGURE 6 The principle of affinity chromatography. (**A**) A complex feed is passed through a column containing beads bearing a covalently attached ligand that recognizes the desired target protein. (**B**) Only the target proteins binds to the ligands. (**C**) The non-target proteins are washed through with buffer. (**D**) The elution buffer contains a counter ligand or another component which encourages desorption of the target protein from the affinity matrix, and the target protein is recovered in a substantially purified and concentrated form from the eluate. (**E**) The affinity matrix is stripped with NaOH and re-equilibrated.

concentrations of the competitor (5–100 mM) and the fact that elution is carried out the functional pH of the target protein, so denaturation does not occur. Non-specific desorption is achieved by changing the pH or ionic strength of the buffer. Decreasing the pH to 2–4 is usually effective, although for proteins that function at low pH ranges an increase in pH may be a more suitable option. Increasing the amount of salt in the buffer disrupts electrostatic interactions, so where protein-ligand interaction is predominantly due to electrostatic bonds, salt gradient elution is a good choice. Where binding is predominantly due to hydrophobic interactions, neither salt concentration nor pH may disrupt binding. Under these circumstances, elution may be facilitated by the introduction of detergents or chaotropic agents into the buffer. An example is the rat biotin-binding protein, which is separated from the immobilized biotin ligand using a combination of free biotin in the elution buffer and one or more protein denaturants.

Although affinity chromatography involves very specific and strong interactions between ligand and target, the interactions are not covalent so column resins can be used for many cycles before replacement. Regeneration of the adsorbent involves a series of washes with reagents such as 0.1 M NaOH that remove all non-covalently bound molecules, theoretically without damaging the ligand itself (called cleaning in place, or CIP), followed by re-equilibration with the initial buffer. Eventually, column media have to be replaced due to fouling (permanent chemical modification of the ligand) and leaching (removal of the ligand from the matrix, either during a process run or during CIP). However, both these problems have been addressed by recent developments in affinity chromatography media. In the case of Protein A affinity chromatography, for example, variants of Protein A have been designed to be more stable in the alkaline

environments used for CIP (Bergander et al., 2004). Alternative ligands for antibody capture have also been described, such as protein G (from *Streptococcus pyrogenes*) which, unlike Protein A, binds IgG3 with high affinity, and protein Z, an engineered variant of protein A. Another alternative ligand is protein L (from *Peptostreptococcus magnus*) which binds specifically to the kappa light chain, (Bjorck, 1998). Due to the higher cost of these reagents, some researchers have attempted to develop chemical and biomimetic ligands derived from peptide libraries, combinatorial chemistry and rational ligand design (Li et al., 1998). Ligands such as 4-mercaptoethylpyridine, 2-mercapto-5-benzymidazole sulfonic acid and certain triazine derivatives cost 50–70% less than Protein A and can survive over 200 cycles of binding, eluting, and CIP with more efficient cleaning reagents such as 0.5–1 M NaOH. They also operate over a greater pH range than Protein A and tend not to leach into the process stream (McCormick, 2005). Non-affinity methods for antibody purification have also proven to be just as efficient as affinity chromatography (Boschetti, 2001; Subramanian, 2005). Such ligands may therefore feature strongly in the future of antibody manufacture.

Ion Exchange Chromatography

Whereas affinity chromatography exploits very specific interactions between the target molecule and an appropriate ligand, other forms of chromatography separate molecules on the basis of general physicochemical properties such as size, charge and hydrophobicity. Therefore, columns may be run in either flow-through mode or retention mode, depending on which is the most efficient for separating the target protein from contaminants. Ion exchange chromatography separates proteins on the basis of their net charge, which as discussed earlier reflects the number and nature of charged amino acid residues on the protein as well as the pH of the buffer (Boschetti and Girot, 2002). The ability to control the polarity and magnitude of a protein's charge by varying the pH is exploited in ion exchange chromatography for the selective adsorption of target proteins onto a resin derivatized with charged groups. Anionic and cationic resins of varying strengths may be used to adsorb proteins of the opposite charge. Some examples of anionic and cationic exchangers are listed in Table 2.

The process for ion exchange chromatography is similar to that described for affinity chromatography with important differences in column capacity and elution mechanics. Initially, the column is equilibrated with a low-ionic-strength buffer containing ions of opposite charge to the resin (Fig. 7). These counter-ions are displaced by charged molecules in the feed stream, which adsorb to the resin. When run in retention mode, the strength and selectivity of binding between the target protein and the resin are optimized by adjusting the ionic strength and pH of the buffer, and the flow rate through the column, such that maximum retention of the target is achieved. In flow-through mode, these conditions are adjusted to optimize the retention of particular contaminants. However, since many proteins will share the same charge profile and pI value as the target, numerous competing molecules will cosegregate in the appropriate fraction. In retention mode, this means that the true binding capacity of the column for the target protein is actually below the theoretical maximum. This operating capacity for the target, in the presence of impurities, is known as the dynamic capacity.

Elution from an ion exchange column is achieved by washing with buffers of gradual or stepped increases in ionic strength, or gradual or stepped changes in pH in the appropriate direction. The principle is very much the same as that employed for the elution of the adsorbed fraction from affinity columns when the affinity of target and

TABLE 2 Some Examples of Anionic and Cationic Exchangers Used in Chromatography

Anion exchangers	Functional group	Comments
Diethylaminoethyl (DEAE)	$-O-CH_2-CH_2-N^+H(CH_2CH_3)_2$	Weak
Quaternary aminoethyl (QAE)	$-O-CH_2-CH_2-N^+(C_2H_5)_2-CH_2-CHOH-CH_3$	Strong
Quaternary ammonium (Q)	$-O-CH_2-CHOH-CH_2-O-CH_2-CHOH-CH_2-N^+(CH_3)_3$	Strong
Cation exchangers	Functional group	Comments
Carboxymethyl (CM)	$-O-CH_2-COO^-$	Weak
Sulfopropyl (S)	$-O-CH_2-CHOH-CH_2-O-CH_2-CH_2-CH_2SO_3^-$	Strong
Methylsulfonate (M)	$-O-CH_2-CHOH-CH_2-O-CH_2-CHOH-CH_2SO_3^-$	Strong

ligand is determined mostly through electrostatic bonds. The main difference is that in affinity chromatography, the selectivity of binding is such that only one elution fraction is generally required, whereas in ion exchange chromatography is it possible to produce a number of different fractions by stepwise elution. The resolution of ion exchange chromatography is influenced by the sample load, linear flow rate and slope of the elution gradient. The best results are obtained by adjusting gradient volumes to the resolution required.

As mentioned above in the filtration section, a more sophisticated approach to ion exchange chromatography is the use of microporous membranes with built-in ion

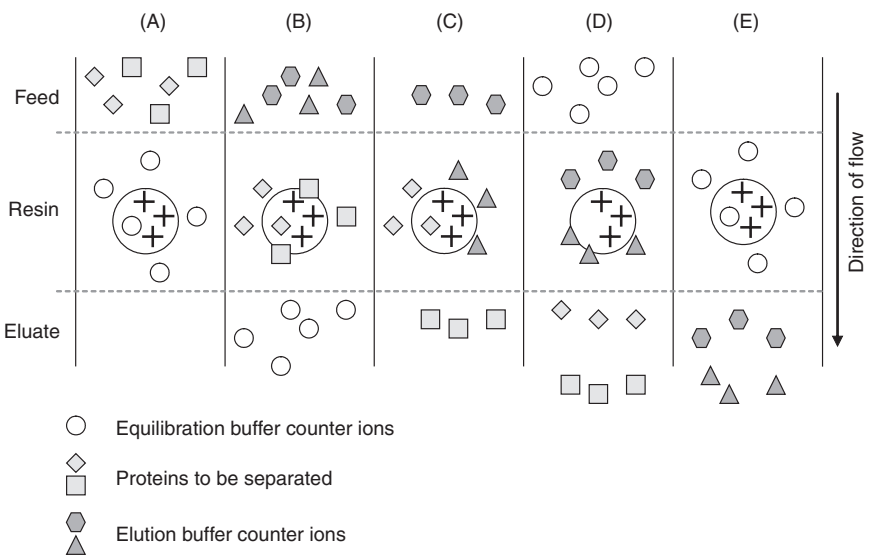


FIGURE 7 The principle of ion exchange chromatography, using a strong cation exchange resin in retention mode. (**A**) Initially, the positively charged resin is occupied by negative counter-ions in the equilibration buffer. (**B**) As the feed moves through the column, negatively charged (acidic) proteins adsorb to the resin and displace the negative ions, while positively-charged (basic) proteins flow through. (**C**) At the start of desorption, gradient ions are added to the column in the elution buffer which disrupt the weakest interactions, so weakly acidic proteins are eluted first. (**D**) Desorption proceeds stepwise, with the most acidic proteins eluted in the last fraction. (**E**) The column is then cleaned in place and regenerated with equilibration buffer.

exchange capacity (Gosh, 2002; Gottschalk et al., 2004; Gottschalk, 2006b). Column chromatography often involves long separation times, and the use of membrane adsorbers can reduce process times by 100-fold compared to conventional chromatography while maintaining a flow rate of 20–40 bed volumes per minute (Zhou and Tressel, 2006). Figure 8A shows some standard chromatographic media on the surface of a Sartobind Membrane Adsorber. More than 95% of binding sites are found inside conventional beads making them inaccessible. In contrast, binding sites on Sartobind membranes are found on a homogeneous film approximately 0.5–1 μm in thickness on the inner walls of the reinforced and cross-linked cellulose network. Diffusion time in such adsorbers is negligible because of the large pores and the immediate binding of target proteins to the ligands. The fluid dynamics of conventional beads and membrane adsorbers are compared in Figure 8B. Membrane adsorber technology is described in more detail in Chapter 22 of this volume.

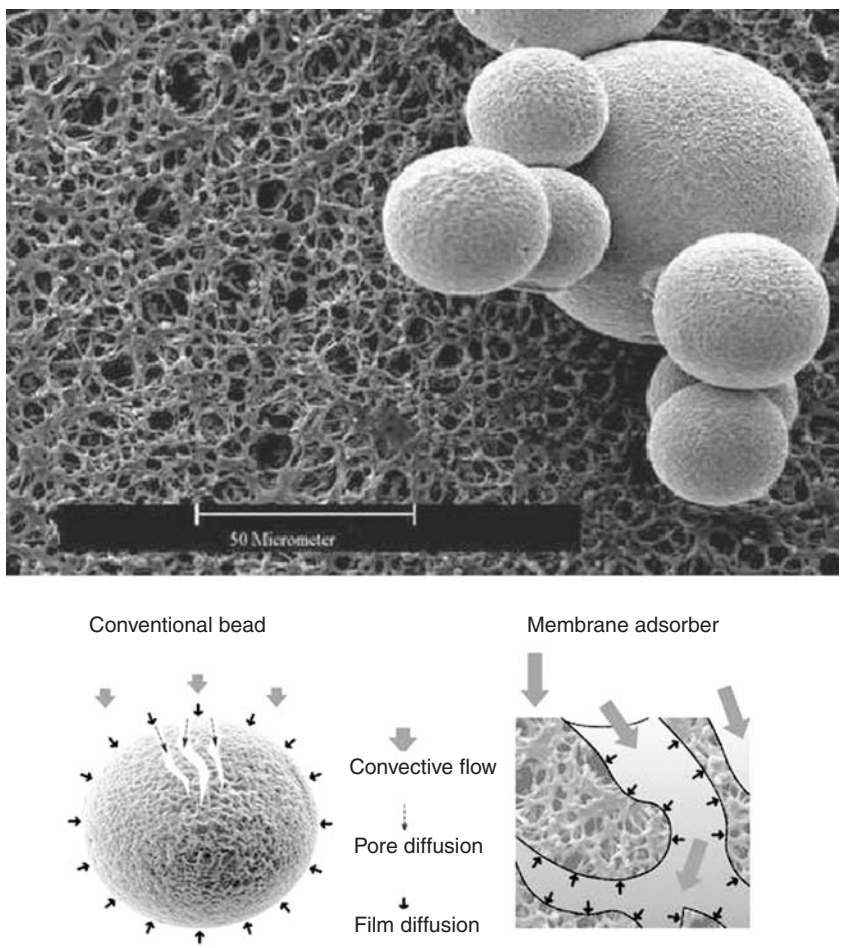


FIGURE 8 (**A**) SEM showing standard ion exchange chromatography beads on the surface of a Sartobind Q membrane. Even at 500-fold magnification, pores in beads cannot be seen, whereas the membrane pores are easily visible. (**B**) Transport phenomena occurring in conventional beads and in membrane adsorbers. *Source*: After Gosh, 2002.

Chromatography Methods that Exploit Hydrophobic Interactions

Many proteins are hydrophobic in nature due to the predominance of non-polar amino acid residues on the surface. Even proteins that are polar and therefore stable in aqueous solvents may have hydrophobic patches that allow them to interact with other proteins with similar characteristics. The basis of such interactions is the increase in entropy that occurs when (polar) solvent molecules are excluded from the interface. This can be exploited as a chromatography technique if a hydrophobic resin is used in concert with a strongly polar solvent—that is, a concentrated salt solution—the chromatographic equivalent of "salting out" (see above). Hydrophobic proteins then increase the entropy of the system by interacting preferentially with the hydrophobic resin.

Like ion exchange chromatography, HIC involves the reversible adsorption of proteins to a resin and elution using a buffer which disrupts such interactions (Jennissen, 2002). In this case (Fig. 9), the resin is equilibrated in a high-salt buffer and the feed stream similarly adjusted to high ionic strength so that proteins bind preferentially to the resin. Desorption is achieved by stepwise reductions in the salt concentration of the elution buffer, sometimes in combination with a gradual increase in the concentration of an organic solvent such as ethanediol which encourages hydrophobic proteins back into solution. The most suitable ligands for hydrophobic interaction chromatography are C_2–C_8 alkyl groups and phenyl groups, which undergo hydrophobic interactions with most proteins but are not so hydrophobic that extreme conditions are required for elution (see below). As in ion exchange chromatography, the stepwise

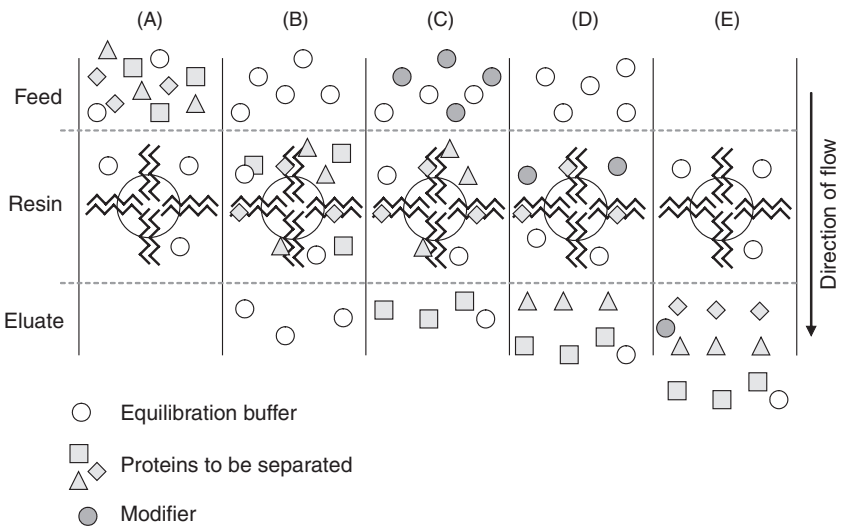


FIGURE 9 The principle of hydrophobic interaction chromatography running in retention mode. (**A**) Initially, the hydrophobic resin is unoccupied, and its interaction with the high salt buffer is energetically unfavorable. (**B**) As the feed moves through the column, hydrophobic proteins adsorb to the resin and displace salt ions, thereby increasing the entropy of the system. Meanwhile, polar proteins interact with the solvent and are washed through. (**C**) At the start of desorption, the amount of salt in the buffer is reduced, allowing the least hydrophobic proteins back into solution such that they are eluted first. (**D**) Desorption proceeds stepwise, with the most hydrophobic proteins eluted in the last fraction where the buffer has a very low ionic strength and may be supplemented with a weak organic modifier. (**E**) The column is then cleaned in place and regenerated with equilibration buffer.

modification of elution buffer composition results in fractions containing sequentially more hydrophobic proteins. The resolution of HIC is influenced by the sample load, linear flow rate and slope of the elution gradient, and can be optimized by decreasing the flow rate and increasing the gradient volume.

RPC is a related technique which also uses a hydrophobic interaction matrix to separate proteins. The main difference is that the resins used for RPC are much more hydrophobic than those used in HIC (e.g., C_{10}-C_{18} alkyl groups) and the elution solvents need to be stronger and usually denature the proteins which are eluted (e.g., acetonitrile, isopropanol, etc.). For this reason, RPC is rarely used for the industrial scale processing of biopharmaceuticals unless the target protein is a polypeptide or small protein equivalent to the size of insulin or below. Large scale RPC is more widely used for the preparation of small molecule drugs, such as antibiotics.

Size Exclusion Chromatography

Size exclusion chromatography (SEC), also known as gel filtration, is distinct from the chromatographic methods described above because it does not depend on selective absorption and desorption. Instead, the principle underlying SEC is the sieving of molecules by size as they percolate through the resin (Eriksson, 2002). The column is packed with inert beads whose surfaces are covered with pores. The size selectivity of the resin depends on the size of those pores, since larger molecules cannot enter the pores and are eluted rapidly, whereas molecules smaller than the pore size will become trapped, and will move through the resin more slowly (Fig. 10). This is known as molecular exclusion, and is distinct from the other forms of chromatography because there is no chemical interaction between the proteins in the sample and the resin.

Resins are available which separate molecules within particular size ranges (Table 3). SEC is therefore used for the fine fractionation of molecules by size, rather in the same way that gel electrophoresis exploits the sieving potential of agarose and polyacrylamide gels. Indeed, many of the SEC media available commercially are based on agarose, polyacrylamide and other polymers. An important concept in SEC is that the separation medium is the pores on the beads and not the beads themselves. Therefore, 9–99% of the column volume remains unused in any operation, and feed volumes must be

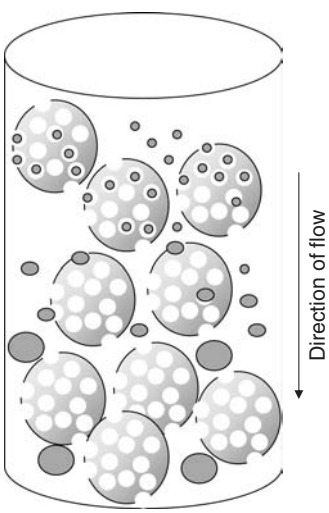


FIGURE 10 The principle of size exclusion chromatography (gel filtration). A column is packed with inert beads containing pores of a certain size. As the feed percolates through the column, small molecules become trapped in the pores and are delayed, whereas molecules unable to fit in the pores travel rapidly through the gaps between beads. Different media are used to separate molecules within different size ranges.

TABLE 3 Some Examples of Size-Exclusion Chromatography Media

Name	Substrate	Size range (M_r)
Bio-Gel P-2	Acrylamide	100–1800
Sephadex G-25	Dextran	1000–5000
Sephacryl S-100 HR	Dextran-acrylamide	1000–100,000
Fractogel TSK HW-55	Hydrophilic vinyl	1000–1,000,000
Superdex 75	Agarose-dextran	3000–70,000
Sephacryl S-200 HR	Dextran-acrylamide	5000–250,000
Sephacryl S-300 HR	Dextran-acrylamide	10,000–1,500,000
Sephacryl S-400 HR	Dextran-acrylamide	20,000–8,000,000
Fractogel TSK HW-65	Hydrophilic vinyl	50,000–5,000,000

adjusted accordingly, representing a significant bottleneck. For this reason, SEC is often the very final stage in biopharmaceutical purification, and is used to separate the target protein from very similar molecules such as degradation products and multimers. The most important variables in SEC are the column length and linear flow rate. Slow mass transfer of macromolecules can cause peak broadening and loss of resolution, which can be addressed by reducing the flow rate.

SEC with resins suitable for separating molecules in the lowest size ranges ($M_r < 5000$) is used to separate macromolecules from low molecular weight compounds, and is thus useful for desalting or rebuffering protein samples. This is an alternative to filtration-based methods, such as diafiltration and reverse osmosis, and has a much higher capacity and flow rate than SEC running in fine separation mode. Sample volumes in buffer exchange may reach up to 30% of the column volume, compared to the 1–3% possible with fine separations (Jagschies, 2006).

LOW TECHNOLOGY TECHNIQUES USED IN POLISHING

Polishing refers to the last stages of purification, the outcome of which is a pure and homogeneous product ready for fill and finish. As well as high-technology filtration and chromatography methods which can be used to purify, concentrate and eliminate viruses from the finished product, there are also a number of lower-technology methods which are economical to apply at this stage. Crystallization and lyophilization are considered as examples below.

Crystallization

Crystallization is the separation of a solute from a supersaturated solution (mother liquor), achieved by encouraging the formation of small aggregates of solute molecules, which then grow into crystals. The crystallization process involves the formation of a regularly-structured solid phase, which impedes the incorporation of contaminants or solvent molecules, and therefore yields products of exceptional purity (Klyushnichenko, 2003). It is this purity which makes crystallization particularly suitable for the preparation of pharmaceutical proteins, coupled with the realization that protein crystals enhance protein stability and provide a useful vehicle for drug delivery, as has been demonstrated with various protein drugs including antibodies (Yang et al., 2003). Protein crystallization has been developed into a proprietary technology for drug

stabilization and delivery by companies such as Altus Pharmaceuticals Inc., Cambridge MA (who have various crystalline hormones, replacement enzymes and other protein drugs in late stage clinical development) and Genencor International Inc. (who have received patents on technology for industrial-scale crystallization). The advantages of crystallization as a final purification and concentration step in clinical manufacturing processes include the following:

1. Large scale crystallization can replace some of the more expensive purification steps in the manufacturing process, making the whole process more affordable.
2. Because reactions proceed very slowly if at all in the crystalline state, interactions between molecules are significantly retarded making crystals the ideal way to store and administer mixtures of biological macromolecules.
3. Solid crystalline preparations can easily be reconstituted into very highly-concentrated formulations for injection, which is particularly useful when intended for subcutaneous administration. The high concentrations required (100–200 mg ml^{-1}) are difficult to achieve in liquid formulations because of aggregation.
4. Protein crystals may be used as a basis for slow release formulations in vivo. Characteristics of the crystal such as size and shape, degree of cross-linking and the presence of excipients can be manipulated so as to control the release rate.

There are numerous crystallization methods, including evaporative, cooling, precipitation, melt and super-critical crystallization, each with specific processing methods and apparatus requirements. Evaporative, precipitation and cooling crystallization are the most suitable for highly soluble products, including proteins. However, the large-scale crystallization of proteins requires dedicated approaches because protein molecules are large, and sometimes easily degradable, so they require carefully designed processes (Lee, 2003).

Crystallization is driven by supersaturation of the mother liquor, which may be achieved by cooling, by evaporation of solvent or by mixing two reactants or solvents. In all these cases the actual concentration of the target molecule becomes higher than the equilibrium concentration, and a driving force for crystallization is achieved. Crystallization begins with nucleation, and nuclei then grow by a combination of layered solute deposition and agglomeration caused by random collision. Crystal growth is opposed by the continual dissolution of the solid phase, but conditions are chosen that favor growth over dissolution. The interplay of all these processes determines the crystal size distribution of the solid, which is an important component of the product specification since it determines the separability of particles, and how they respond to washing and drying. Because this is such an important property, the kinetic processes underlying crystallization have been extensively modeled, and can be predicted by using the population balance equation, which describes how the size distribution develops in time as a result of various kinetic processes.

Crystallization can be triggered in several ways, and these are divided into primary and secondary nucleation mechanisms. In primary nucleation, the solid phase forms spontaneously from a clear mother liquor. In cases of heterogeneous nucleation, crystals nucleate around tiny contaminating particles that are present in the solution. In a perfectly pure mother liquor there is no such substrate, and clusters of solute molecules are thought to form randomly, simply through the statistical fluctuation in their distribution (homogeneous nucleation). Secondary nucleation occurs in mother liquor where crystallization is already in progress. It reflects the formation of tiny crystal fragments through the collision of existing crystals with each other, or with the walls of the

crystallizer, which then serve as nuclei for the growth of new crystals. Most crystals are formed by primary nucleation during the initial phase of evaporative or cooling crystallization. When these have grown to form larger crystals, secondary nucleation becomes the predominant source of new crystals. For proteins with low solubility, primary nucleation tends to remain the dominant mechanism throughout crystallization because supersaturation remains high enough, and the crystals tend to be small and therefore not so prone to fragmentation.

Once nuclei have formed, crystal growth occurs by a number of mechanisms. Uniform growth from solution involves the diffusion of additional solute molecules towards the crystal/solution interface followed by their integration into the crystal surface, which creates a local concentration gradient that encourages further crystal growth, at least for highly soluble molecules. Further, non-uniform growth can occur by agglomeration, which is caused by the collision and subsequent cementing together of small crystals. Under supersaturating conditions which favor uniform crystal growth by solute deposition, the cementing of attached particles occurs through the deposition of additional solute molecules that bind the attached crystals together in a common lattice. If there is no supersaturation, attached particles tend to break apart again. The major processes by which collisions and subsequent agglomeration occur include Brownian motion, laminar or turbulent flow within the crystallizer, and gravity settling. The development of crystallization as a technique in biopharmaceutical production has been most recently reviewed by Peters et al. (2005).

LYOPHILIZATION

Lyophilization or freeze-drying in pharmaceutical manufacture is a process in which a pure dissolved product is frozen and then dried by exposure to conditions that cause sublimation of the ice (Oetjen, 2000). With pure water as the solvent, reducing the pressure to less than 0.6 atm in a vacuum chamber is sufficient to prevent the formation of liquid water when the frozen product is heated above freezing point. The general approach is therefore to freeze the product and place it on a heated shelf in a vacuum chamber. Once the chamber is evacuated to below 0.6 atm, the temperature is raised to just above the freezing point of water resulting in the sublimation of the ice. At this stage, it is the bulk solvent that sublimates, and this may represent 10–100 times the volume of the dry product depending on its initial concentration. This process is known as *main drying*. Freezing and main drying are usually followed by a *secondary drying* step involving the desorption of water bound to the solid. For pharmaceutical proteins, this means water molecules attached to the protein via hydrogen bonds. Such water molecules form a monolayer around the protein and have distinct properties to the bulk solvent. They may constitute as little as 5–10% of the volume of the dried product, but even this amount of water can facilitate some enzymatic reactions that would cause protein degradation. Once this water has been removed, the lyophilized solid is packaged under vacuum to prevent any further exposure to water until ready for formulation.

Lyophilization, like crystallization, is a useful final-stage procedure in biomanufacturing because it provides a way to prevent the reactions that normally occur in solution. Therefore, the process can enhance the stability of a protein pharmaceutical and allow it to be stored for prolonged periods at ambient temperatures without fear of degradation or loss of activity. Lyophilization is relatively expensive to carry out for large-scale processes but the benefits of increasing drug longevity and eliminating the requirement for a cold chain can far outweigh the initial costs.

For pharmaceutical products, the lyophilization process should not impede reconstitution of the drug for formulation. For other products, such as cells, it is necessary to use cryoprotectants to prevent damage to cellular structures and membranes during freezing. For protein drugs, however, the tendency of nascent ice crystals to form pores in the solid product is actually beneficial, since this facilitates access for water molecules when the product is reconstituted and allows it to dissolve rapidly. This is important to maintain the product's structural integrity and biological activity.

CHALLENGES AND OPPORTUNITIES

Expression Platform Diversity

Traditional biomanufacturing has concentrated on a small number of well-characterized upstream production systems, and downstream processes have been developed largely with these systems in mind. The most popular systems are bacterial cells (*Escherichia coli*) for the production of simple proteins (Choi and Lee, 2004; Baneyx and Mujacic, 2004) and mammalian cells (CHO cells and a small number of alternative rodent or human cell lines) for the production of complex proteins and glycoproteins (Wurm, 2004). A few approved pharmaceutical proteins are made in yeast or in cultured insect cells. One striking characteristic of biomanufacturing in the twenty-first century is that a large number of novel production systems are being explored. This reflects advances in underlying gene transfer and expression technology and perceived advantages of economy and production scale made possible by the adoption of new production systems, especially those based on transgenic animals and plants (Dyck, 2003; Twyman et al., 2005). The industry's faithful relationship with microbes and mammalian cells is being tested by limitations of production capacity which are becoming evident, and the costs associated with building, testing and validating new fermenter-based production trains.

The benefits and limitations of bacterial production systems in terms of downstream processing technology are well understood. The recombinant protein is usually expressed as an intracellular product that must be recovered by cell disruption, often accomplished by concussion in a ball mill or by the application of shear forces for example, in a homogenizer or colloid mill. Some products are expressed as soluble proteins, and in such cases microfiltration is used to remove cell debris and fines prior to capture. However, many recombinant proteins aggregate to form insoluble inclusion bodies, and in these cases continuous centrifugation may be used to separate the dense inclusion bodies from other particulate matter. Solubilization using strong denaturing buffers, followed by protein refolding and renaturation are required to recover an active product, which can be time consuming and difficult to implement on a large scale.

Similarly for eukaryotic cells, downstream processing steps have become well established. The general strategy is to secrete the product into the fermentation broth, so clarification involves separation of cells from the liquid broth by centrifugation and/or filtration and product capture from the supernatant/permeate. While yeast cells are tough, mammalian and insect cells are fragile, so the pressure must be carefully controlled to avoid lysis. Depth filtration is widely used at this stage.

The recent proliferation of new upstream systems has challenged downstream processing to come up with complementary processing methods. New systems include the production of recombinant proteins in cultured plant cells, animal milk, serum or urine, silkworms, hens' eggs, and whole plant tissues such as leaves and seeds. Each of these systems presents new potential advantages in terms of production benefits, but also

presents new challenges to downstream processing (Nikolov and Woodard, 2004; Menkhaus et al., 2004).

There is no danger of cell lysis when proteins are expressed in animal body fluids, but each introduces a unique set of contaminants that must be dealt with early in the production train. For example, milk contains a number of endogenous proteins that compete with the recombinant product, as well as fat globules, casein micelles and soluble lipids which can foul filters and membranes leading to poor process efficiency. The clarification of animal milk should therefore include a centrifugation step to remove fat droplets followed by a dedicated membrane filtration to remove micelles (Baruah et al., 2003). Alternatively, it is possible to precipitate micelles although this can lead to the target protein becoming trapped in micelle aggregates thus reducing recovery rates. Similar principles apply to the recovery of proteins from hens' eggs—the egg white is highly viscous and unsuitable for processing without taking steps to reduce viscosity, which can be achieved through the acid precipitation of ovomucin (Rapp et al., 2003).

Plant tissue also presents challenges in the early stages of downstream processing (Menkhaus et al., 2004). Seeds must be ground or milled to flour to allow the solubilization of the target protein and maximize recovery. The processing of leaves must take into account the possibility of contamination with oxidizing agents and proteases as the tissue is shredded or pressed to remove the green juice. In small scale processes, this can be addressed using antioxidants and protease inhibitors in the extraction buffer, but this would be too expensive on a large scale and processes must be adapted to isolate the target product rapidly from the most damaging contaminants. In the case of plant-derived pharmaceutical proteins, this may well involve the use of dedicated, chemically-active membrane filters to remove particulates, fines and also specific target contaminants such as proteases, phenolics and oxalic acid, all of which can cause damage to proteins in solution.

Integrated Process Design

Each process train should be designed de novo based on the optimum arrangement and juxtaposition of different operational units to suit both the upstream production system and the product. There is no perfect system suitable for all products and platforms, but the general design of a process should take into account certain rules of thumb reflecting the economy, duration and overall safety of production (Fish, 2004). In general, it makes economic sense to place the least expensive processes early in the production train, as these will bear the greatest contaminant load and the largest feed volumes. These early processes should aim to remove the bulk of the contaminants, and all of the specific contaminants that might poison operational modules further downstream through membrane fouling etc. The early steps should also aim at volume reduction, thereby increasing the productive output of the later and more expensive separation steps. In the case of chromatography, for example, the cost of column resins and their frequency of replacement is the most significant economic factor. The lower the input volumes and the less often the resins have to be replaced or recharged due to fouling, the more economical the process.

For optimal process and cost efficiency, there should be as few process operations as possible and each should exploit, to the maximum extent, the physical and chemical differences between the recombinant product and the set of target contaminants that are to be removed. These processes should be ordered in a rational manner so that consecutive separation steps exploit different separative principles

(orthogonal separation). Most importantly of all, however, one needs to move away from the idea of separate and independent operative units and towards the concept of an integrated production train (Gottschalk, 2005c). The optimization of individual units can lead to improved output from a particular unit, but may reduce the overall efficiency of production if the impact of such changes on downstream units is not considered. Process engineering and modeling should be used to develop and refine the simplest, most efficient and most robust processes, thus helping to overcome some of the current bottlenecks.

As an example, we can apply this concept to antibody manufacture (Fig. 11). It would be advantageous to employ a capture phase that utilizes the high dynamic capacity of affinity resins, followed by a filtration train for polishing, taking advantage of the properties of charged membranes (Gottschalk, 2005b). Processes requiring only two columns and a filtration train would minimize costs without affecting efficiency, especially if the filtration train employed smart membranes appropriate for the removal of specific contaminants. The idea of polishing before capture is heretical, but in this example it would protect the Protein A column from fouling and therefore extend its useful lifetime and operative capacity.

Economic Barriers—Upscaling and Technology Replacement

The biopharmaceutical industry is dominated by the fixed costs of production, which include the planning and development of new manufacturing processes and facilities, and the costs associated with regulatory compliance. Over the last 20 years, the spiraling costs of production have been addressed, to a certain extent, by increasing productivity. Certainly the output of key products such as monoclonal antibodies has increased over 100-fold since the first industrial processes were approved. The vast majority of this productivity increase has occurred in the upstream part of the production train, through the development of better performing cell lines, and the use of improved media, additives

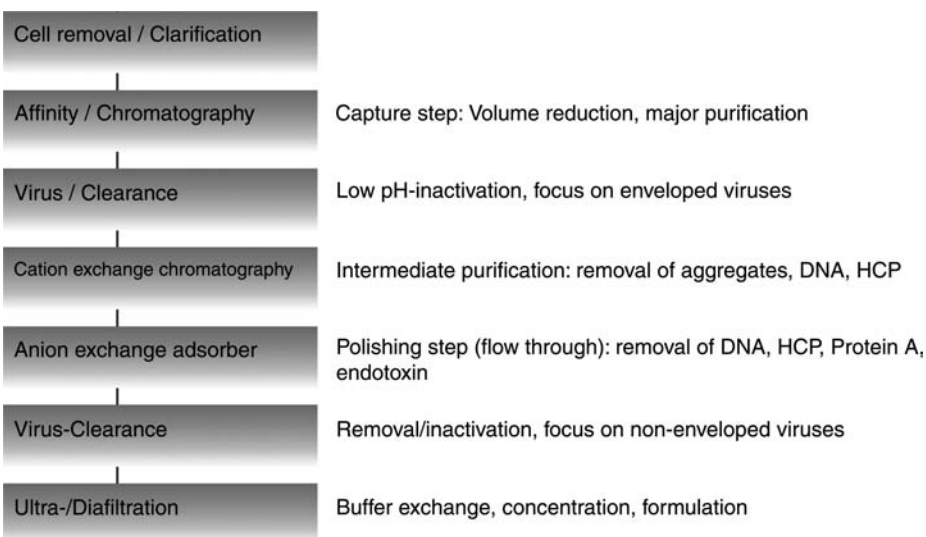

Cell removal / Clarification
Affinity / Chromatography
Capture step: Volume reduction, major purification
Virus / Clearance
Low pH-inactivation, focus on enveloped viruses
Cation exchange chromatography
Intermediate purification: removal of aggregates, DNA, HCP
Anion exchange adsorber
Polishing step (flow through): removal of DNA, HCP, Protein A, endotoxin
Virus-Clearance
Removal/inactivation, focus on non-enveloped viruses
Ultra-/Diafiltration
Buffer exchange, concentration, formulation

FIGURE 11 A generic manufacturing strategy for monoclonal antibodies. *Abbreviation*: HCP, host cell proteins.

and reactor design. Progress in downstream processing has been relatively slow in comparison, and this is perhaps the biggest challenge facing the industry at the current time. Ultimately, there are only a few ways to remove bottlenecks and increase the productivity in biomanufacture. These include up-scaling current process technology, replacing current processes with more efficient ones and the increased adoption of disposables. These three trends are discussed below.

Up-Scaling

The high-end technologies of downstream processing such as chromatography are reaching their limits, both physically and economically. For example, a relatively straightforward approach to scaling up any form of chromatography is simply to increase the column diameter. The sample load is then increased proportionately, so that the linear flow rate is maintained but the overall throughput increases. Unfortunately, scaling up in this manner has a number of unpredictable effects, including zone broadening, which can reduce the overall efficiency and resolution of the separation. The largest chromatography columns in current use are some 2–3 m in diameter, which is about the maximum that can be tolerated without running into operational problems (Jagschies, 2006). We are reaching the point at which increasing the scale of each unit operation no longer leads directly to an increase in productivity, and this law of diminishing returns as the footprint of each operation increases cannot be sustained.

Further gains can be made by increasing the dynamic capacity of the resins and the linear flow rate, leading to the optimization of throughput without further scaling. However, at some stage every chromatography system will reach its optimal operational performance, thus making further increases in productivity reliant on new technological developments. Even if continued up-scaling were possible without compromising efficiency, the cost of the resins and the equipment itself soon becomes unsupportable. Currently, downstream processing accounts for 50–80% of production costs, but as ever higher capacities are demanded, this proportion is likely to increase; downstream processing is thus suffering from the lack of technologies that are robust and scalable as well as affordable, and that do not compromise either productivity of product safety. In addition, the up-scaled processes need to be validated before they are put to use, which reveals further sets of challenges as summarized by Sofer (1996) and are discussed extensively in Sofer and Zabriskie (2000) and Rathore and Sofer (2005).

High- vs. Low-Tech Processes

Instead of striving for scientific breakthroughs to make high-end technologies more efficient and scalable, an alternative approach is to revisit some of the robust low-end methods which are the staple of the chemical industry and are already being applied in the manufacture of small-molecule drugs (Gottschalk, 2006a). Unlike other monologues on downstream processing, which focus on high-end technologies, equal space has been given in this chapter to approaches such as extraction, precipitation and crystallization. These techniques may be one key to addressing the capacity crunch in downstream processing, as they are reliable, robust, scalable and above all, economical. There is a perception that forsaking chromatography for such methods would cause regulatory issues, but this is probably unfounded. Since these approaches are already used in the pharmaceutical industry and they can be applied under cGMP standards, there seems no reason why they should not eventually be accepted for use in biopharmaceutical production. The development of crystallization as a polishing and formulation method for

protein pharmaceuticals by companies such as Altus Pharmaceuticals Inc. shows that such technologies are not only viable and profitable, but also offer innovative solutions to problems such as drug delivery that have not been addressed by other methods.

Disposable Concepts

Another major challenge in downstream processing is the requirement for cleaning and validation, both of which are expensive in terms of the procedures themselves and the downtime they cause which wastes valuable production time (Sinclair and Monge, 2004; 2005). Increasingly, this is being addressed by supplementing or even replacing permanent fixtures and steel piping with disposable modules. The higher cost of such modules compared to the single purchase and installation cost of a permanent fixture is offset by the elimination of CIP and SIP (steaming-in-place) procedures, the validation of cleaning routines and the associated record keeping (Sinclair and Monge, 2002). Hard-piped components require cleaning, and the success of cleaning must be validated. For filters and resins, cleaning and regeneration can be expensive and time consuming, and validation equally so. Also, CIP procedures involving harsh chemicals inevitably lead to the degradation of chromatography media and membrane chemistry, ultimately necessitating replacement.

The rise in disposable module use marks an important paradigm shift in downstream processing, which may ultimately lead to fully modular process trains where every unit operation is disposable (Gottschalk, 2005c). This would achieve one of the holy grails in downstream processing: a continuously operative and maintenance-free process train, where exhausted or malfunctioning units can simply be swapped for new ones, and where there is no need for cleaning or validation and therefore zero downtime. Each production cycle would be carried out with components that have never been in contact with previous batches, thereby avoiding all forms of cross-contamination. Disposable modules are now a staple part of laboratory work (e.g., spin columns, disposable filters) but thus far only disposable filter modules have become commonplace in industrial scale processes. In the future, we are likely to see the rapid adoption of disposable chromatography membranes, which have been shown to work as efficiently as columns but with a much smaller footprint (Zhou and Tressel, 2005; 2006). For example, a disposable 0.5-L membrane has been shown to remove process contaminants from a 1000-L feed stream as efficiently as a stainless steel column packed with resin that is 100 times larger. Taking monoclonal antibody manufacture as a model system for cost analysis, it has been estimated that membrane chromatography gains an equal footing to traditional columns when loaded with 2 kg of antibody, but falls to 20% of the cost of columns when loaded to 10 kg (Warner and Nochumshon, 2003; Hodge, 2004; Mora et al., 2006; Delmdahl, 2006). However, costs are only one benefit of disposable technology and one must also factor in the additional convenience and reduction in downtime. Polishing applications are therefore likely to be dominated by disposable membranes in 5 years as the cost and efficiency benefits become more apparent (Gottschalk, 2006a).

CONCLUSIONS

In this chapter, we have considered a range of technologies which are currently used or could in the future be used in the downstream processing of protein pharmaceuticals.

The challenges we face today include how to deal with ever increasing upstream productivity and how to adapt to novel production systems, both of which have uncovered bottlenecks downstream. We have explored some of these challenges and their potential solutions, looking at tailoring downstream processes to match particular platform-product combinations, the development of an integrated process concept, and reviewing how technology can be applied in downstream processing to match the upstream demands.

At the current time, downstream processing is heavily reliant on high-technology operations such as chromatography, which is reaching its economic and physical limits. The solution to this problem is to continue with the development of novel technologies such as membrane chromatography, and marry this with the re-examination of some robust low-technology methods such as extraction, precipitation and crystallization, which are all mainstays of the biochemical and pharmaceutical industries, tried and tested for the production of small molecule drugs. Combining this with the use of disposable modules to reduce the length of time spent off production will bring about a critically-needed increase in capacity, and will hopefully remove some of the hurdles which prevent the efficient and high-throughput processing of biopharmaceutical products.

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19
Crossflow Filtration

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INTRODUCTION

Modern ultrafiltration (UF) membranes have their origins in the 1950s when commercially viable reverse-osmosis (RO) membranes were developed for water desalination (Michaels, 1986). The Loeb and Sourirajan's phase inversion process created the first highly anisotropic membranes, which is the basis of UF membranes used today.

The membrane asymmetry created by the Loeb–Sourirajan's process reduced the thickness of the membrane's rejection layer 1000-fold less than that of previously produced symmetric membranes like those used in microfiltration (MF) (Cheryan, 1986; Loeb, 1981). This singular development by Loeb and Sourirajan began the process of development to make it commercially practical to use these membranes in the biopharmaceutical industry today (Cheryan, 1986).

Membrane filters are made in a wide variety of pore sizes. Figure 1 shows the effective pore size for membranes used in RO, nanofiltration (NF), UF, and MF.

Filtration processes may be operated in one of two modes. Flow may be orthogonal (dead-ended or static filtration) or tangential to the filter. Process scale UF applications are typically performed using crossflow or tangential flow filtration, at least at a scale of operation where $> 50\,cm^2$ of membrane is used. For Laboratory R&D scale when less than $50\,cm^2$ of membrane is required, stirred cell and spin filters (centrifuge) are used where the flow is orthogonal to the filter. In crossflow or tangential flow filtration the feed stream flows parallel to the surface of the membrane. A fraction of the feed stream permeates (passes through) the membrane; while the remaining fraction is retained by the membrane and exits as retentate and is recycled back to the feed vessel.

In the absence of solute, flow through the membrane is accurately modeled by the Hagen–Poiseulle Equation, which describes liquid flow through cylindrical pores (Chervan, 1986):

$$J = \frac{\varepsilon \cdot r^4 \cdot \Delta p}{8 \cdot \eta \cdot \Delta x}, \tag{1}$$

where J = liquid flux, (flow rate through the membrane)
ε = membrane porosity,
r = mean pore radius,
Δp = transmembrane pressure,

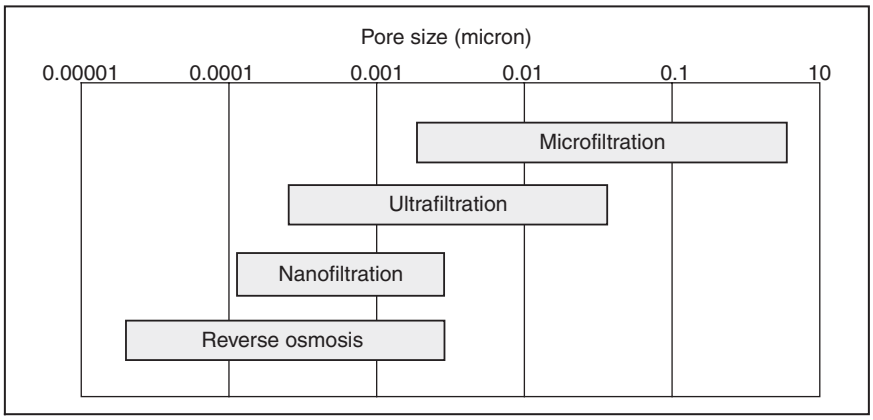


FIGURE 1 Typical pore sizes for membranes used in reverse osmosis, nanofiltration, ultrafiltration, and microfiltration.

η = kinematic liquid viscosity, and
Δx = pore length.

This equation states that the liquid flux is proportional to the transmembrane pressure (TMP) and inversely proportional to the liquid viscosity, which is controlled by the solute concentration and the temperature.

When solute is present in the feed stream, permeating liquid brings solute to the membrane surface by convective flow. As retained solute builds up on the membrane surface a solute layer or cake is formed (often referred to the gel layer). The result of this added solute to the membrane's surface causes an increase in the resistance of flux through the membrane. The thickness of the solute cake is dependent on a number of factors including: (*1*) the rate at which permeating liquid brings solute to the membrane surface, (*2*) the rate at which solute back-diffuses into the feed stream, and (*3*) the hydrodynamic shear of the tangentially flowing stream transporting solute back into the bulk solution. Successful exploitation of membranes in crossflow filtration is largely dependent on effective fluid management techniques (Porter, 1981). "By using hydrodynamic considerations, polarized solutes can be sheared from the membrane surface, thereby increasing the back diffusion and reducing the decline in performance (i.e., reduction in permeation rate)" (Belfort, 1987). Equation (2) has been historically used to describe flux performance in ultrafiltration applications.

$$J_w = k \ln\left[\frac{C_g}{C_f}\right]. \tag{2}$$

where J_w = liquid flux,
C_g = solute concentration at the membrane surface,
C_f = solute concentration of the bulk feed,
k = mass transfer coefficient.

However, as a practical matter, due to solute-membrane interactions it has been found that, as pressure increases, flux may become independent of pressure. When pressure (TMP - average upstream system pressure) increases, the resultant increase in flow through the membrane causes the solute cake (polarized layer) to thicken

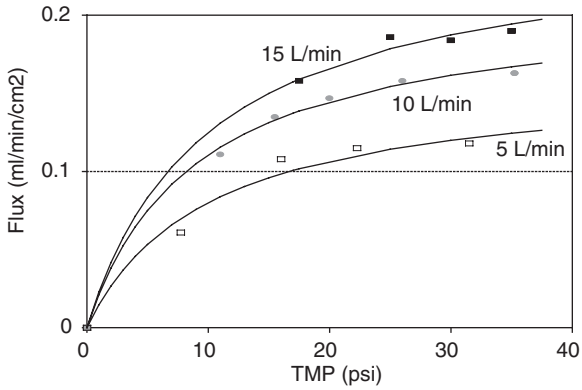


FIGURE 2 Bovine serum flux through a Sartorius Sartocon 0.7 m^2 100 k polysulfone ultrafilter cassette at 3 recirculation flow rates.

proportionally, preventing a concomitant increase in flux. Figures 2 and 3 show examples of this flux versus pressure relationship.

In plots of permeate flux verses solute concentration the mass-transfer coefficient (k) can also be determined from the slope of the line which is proportional to k. Figure 4 shows that k is a function of the crossflow (recirculation flow) velocity, with k increasing as crossflow velocity (flow rate) and shear rate increases, thus reducing the gel layer.

This dependence of the mass-transfer coefficient on the shear (velocity) of the recirculating (crossflow) has been accurately correlated for 10 kDa hollow fiber

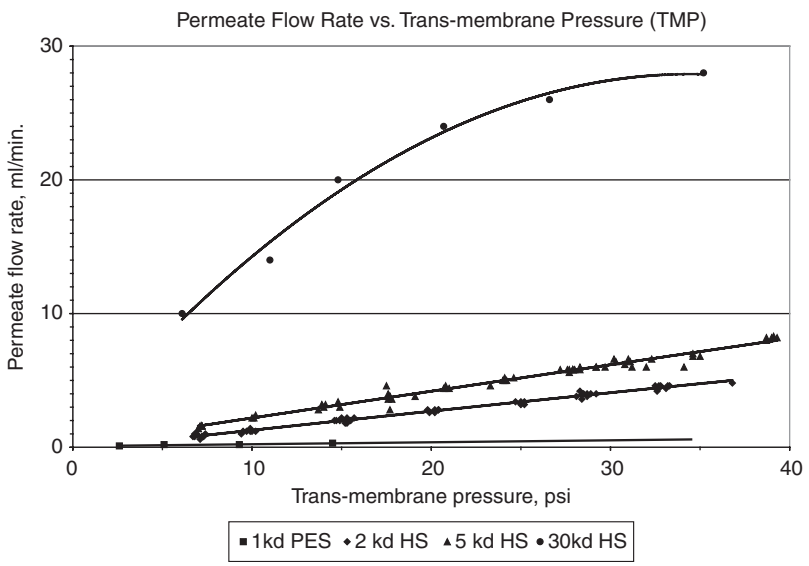


FIGURE 3 Milk diluted 1:5 in saline and filtered through a 1 kDa PES, 2 kDa, 5 kDa, and Milk diluted 1:10 filtered through a 30 kDa Slice 200 ultrafilter cassette membranes, with 200 cm^2 of available surface area at a feed flow rate of 150 ml/min.

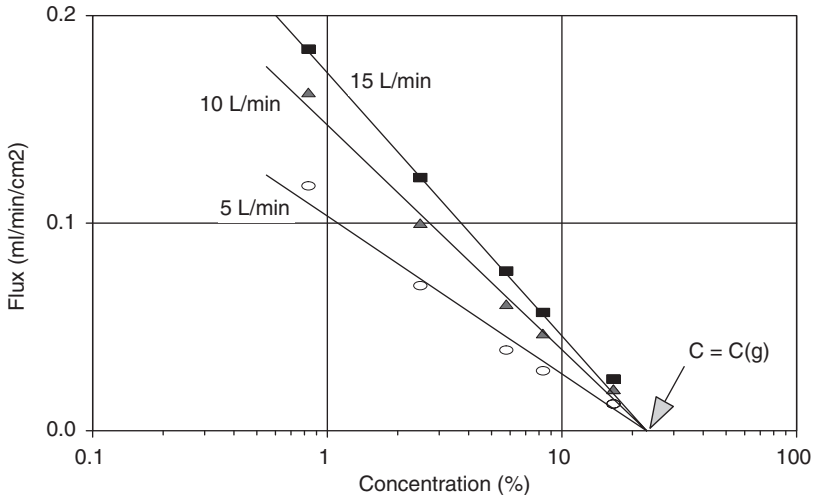


FIGURE 4 Flux vs. concentration for ultrafiltration of bovine serum using 100,000 MWCO polysulfone membrane at three different crossflow rates (Sartorius Sartocon II, membrane area $= 7{,}000\,\text{cm}^2$).

filters in dairy applications (Blatt et al., 1971; Cheryan, 1986). For laminar flow the correlation is:

$$k = 0.816\left[\frac{\gamma}{L}D^2\right]^{0.33} \tag{3}$$

where γ is shear rate, and $\gamma = 8v/d$ for flow through tubes, and $\gamma = 6v/h$ for flow through rectangular channels; v is solution velocity; d is tube diameter; h is channel height; L is length of the membrane flow path; and D is solute diffusivity. When flow is turbulent, the mass-transfer coefficient is proportional to velocity raised to the 0.80 power instead of to the 0.33 power as in laminar flow:

$$k = 0.023\left(\frac{1}{d_h}\right)^{0.20}\left(\frac{\rho}{\mu}\right)^{0.47}(D)^{0.67}(v)^{0.80} \tag{4}$$

where d_h is the hydraulic diameter and equals four times the cross-sectional area divided by the wetted perimeter, ρ is liquid density, and μ is liquid's kinematic viscosity. Because of the greater dependence on velocity when flow is turbulent, improved benefits in flux can be realized when flow is increased. The relationship between flux and velocity for both laminar and turbulent flow is shown in Figure 5.

This shear dependent flux relationship is particularly true for Polyethersulfone (PES) based membranes where considerable membrane fouling occurs. The consequence of protein fouling is the formation of a protein gel that irreversible adheres to the membrane surface. These protein gels become secondary membranes that have protein rejection characteristics that mask the intrinsic properties of the membrane. Additionally, the resistance to flow is also high through these protein gels. Once they are formed they cannot be reversed through the control of the process hydraulics but if the initial recirculation rates are sufficiently high the initial flux values may be higher if there is a strong dependence on the recirculation rate. When proteins do not adhere onto the membrane surface dependence on recirculation velocity becomes markedly diminished as has been reported for regenerated, and modified regenerated cellulose membranes

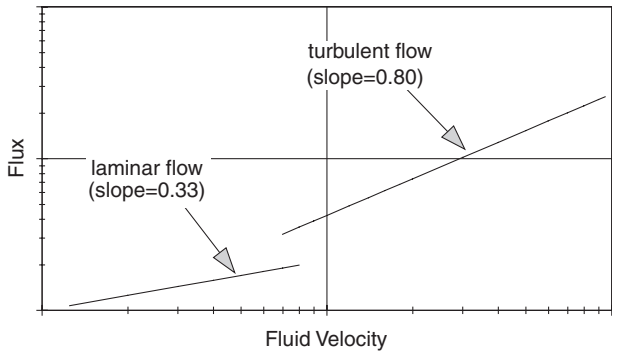


FIGURE 5 Ultrafiltration flux as a function of fluid velocity.

(Dosmar, 2005). This is true because the polarized protein layer does not have as great of a resistance to flow because the interstitial spaces between native protein is greater than that of denatured protein gels. Dependence on surface shear though remains proportional to protein concentration.

Because of these flux dependent parameters, that is, recirculation rates and pressure profiles, it is clear that control and understanding of them is important in the scale-up of any crossflow process.

Some of the conventional dogma associated with ultrafiltration performance no longer seems applicable to the ultrafilters used today.

The requirements for mathematical models that are predictive of membrane performance are that the model is broadly applicable and that formulae used are robust. The predictive mathematical models established in the 1970s were established using 10,000 MWCO hollow fiber polysulfone ultrafilters. The initial work used to establish generally accepted performance profiles was performed in dairy applications on the filtration of whey. This model continued to be applicable as newer polyethersulfone and cellulose triacetate ultrafilters replaced the older polysulfone membranes.

Today, many currently available non-fouling regenerated and modified regenerated cellulose ultrafilters exhibit performance characteristics that fail to adhere to the previously established "gel polarization" model of the past. Membranes with MWCO's 10,000 Daltons and tighter typically behave according to the Hagen–Pasoulle formula and show little flux improvement as recirculation rates are increased, at least at lower protein concentrations. Membranes with MWCO's greater than 10,000 Da do show a strong dependence on the recirculation rate as well as entering into a pressure independent region at higher TMP's. Figure 3 shows and example of flux versus TMP for ultrafilter membranes both greater and lesser than 10 kDa which support this.

Concentration and diafiltration processes are developed at laboratory or process development scale. Successful implementation of these applications is the result of careful analysis of the process hydraulics, (i.e., recirculation flow rates, pressure profiles, retentate flow channel, permeate flux values and membrane surface area) and a process' dependence on shear and pressure. As these processes are scaled up from research scale to pilot scale and again to commercial scale, process engineers are often faced with limited data that at first glance may seem adequate for straight forward scale up, but in fact are lacking critical information. The consequence of failing to recognize these critical parameters may result in predicted scale-up performance that may yield unexpected results at large(r) scale. Shortfalls in performance can have serious economic consequences arising from added labor costs and yield losses.

APPLICATIONS IN THE BIO-MANUFACTURING PROCESS

Traditional applications for crossflow filtration can be grouped into three basic types of operations; concentration, fractionation, and diafiltration.

Concentration is the process of decreasing the solvent content of a solution to increase the concentration of solute. Typical concentration applications include the separation of cells from cell culture broth and recovery of the concentrated cell mass, the dewatering and subsequent concentration of proteins and peptides, and the harvesting of viruses.

Fractionation may be described as a separation process where one of solutes is separated or fractionated, one from the other by means of passing the smaller solute through the membrane while retaining the larger solute in the feed solution. Examples of membrane fractionation are depyrogenation, lysate clarification, and molecular-weight separation of poly-dispersed polymers like proteins and carbohydrates. Depyrogenation of low-molecular-weight solutions like buffers and pharmaceutical preparations is accomplished by retaining the contaminating LAL reactive material in the retentate and allowing the pyrogen-free product to pass through the membrane (Brown and Fuller, 1993). Clarification of cell lysates is the fractionation or separation of cellular debris from the products of interest. Cell lysates can be very fouling in nature, and the success of the operation is very much affected by the selection of the appropriate membrane, pore size, and operating parameters (Sartorius, unpublished data).

Diafiltration is a term combining the words dialysis and filtration. In most dialysis operations the goal is to either exchange one buffer system for another or to wash as in fractionation a contaminant from the bulk.

Microfiltration Applications

Harvesting and Clarification

When evaluating crossflow for cell harvesting and clarification processes it is usually necessary to limit the permeate flow rate using either a permeate pump or a control valve. Permeate control is used to limit the rate of solids being brought to the membrane surface via convective flow and allowing the sweeping tangential flow to lift and re-suspend that material into the bulk feed so as to limit pore plugging from occurring. By controlling the TMP, and monitoring the permeate pressure one may be able to determine whether and how quickly membrane plugging is occurring.

All crossflow applications for cell processing are imperically designed and therefore may differ considerably. As a result no particular rigorous equations have been applied consistently for MF processing.

Perfusion (clarification). Cell separation is one of the most difficult process steps to optimize in the production of monoclonal antibodies from recombinant cells.

The purpose of the perfusion process is to extend the cell growth phase of the cells, while either producing more extra-cellular products, or increasing product cell density and yields of the product in the cells. This is done by using a separation media to remove either spent or conditioned media while at the same time adding fresh media to the reactor. It must be noted that there are special requirements for separation devices used in this way. Considering that one is dealing with a cell culture the complete system must be able to be sterilized and aseptically maintained. Bioreactors and their attendant components are sterilized by means of pressurized steam. If the product is cell associated, then the cells are either removed periodically for harvesting, or collected at a predetermined time or high cell density.

Devices for this application must be able to operate, at a constant permeation rates over extended periods of time. This may mean several days to several weeks of constant operation. During the development of this type of application, operating conditions must be established that assure that the microporous structure of the membrane is not altered and damaged during the sterilization process. Additionally, the membrane must not be plugged or fouled by the cells or cell debris and membranes are not fouled buy antifoam during the course of the process. The permeate flux must be well controlled at all times to ensure process consistency. These systems are especially sensitive to pore plugging during the initiation of the process, so to avoid premature failure, the permeate line must be closed until the feed and retentate conditions are established.

Cell Harvesting of extra-cellular products. Crossflow filtration, as a primary separation process, is especially well suited for the harvesting whole cells from a fermentor or bioreactor prior to further down stream processing. Cells harvested by crossflow filtration can be fully washed resulting is high yield recovery of the target product in the pooled permeate.

Use of 0.45 μm or larger pore crossflow filters will generally yield a clean, cell and debris free protein solution. Recirculation rates may range from < 5 to > 10 LPM per m^2 of membrane surface area while the flux will range between 30 and 100 LMH (liters per square meter membrane area per hour).

Two critical parameters in processing whole cells are the pump speed as measured by the revolutions per minute (RPM) and the resulting rotor "TIP" speed and the TMP. If the rotor tip speed is too high, generally considered above 350–450 RPM, the resultant shear induced turbulence may eventually damage or rupture the cells. Thus most crossflow systems use rotary lobe pumps operating at relatively low speeds.

The driving force for the crossflow process is the TMP (see the preceding section). If the TMP exceeds a certain critical value cells may be disrupted due to compression. Evidence of mammalian cell lysis is an increase in the levels of lactose dehydrogenase in the extracelluar media. For mammalian and insect cells TMP values are usually maintained below 6–10 psi. Yeasts and bacteria can tolerate higher pressures. If yeast, fungi, or bacteria are used to produce extra cellular products, then cell concentration and viscosity are usually limiting factors for crossflow.

The use of crossflow microfiltration applied correctly to extra-cellular protein production containing a high percentage of whole cells should result in a cell and cell protein free product solution that can be easily processed in the down stream process train after sterile filtration.

Intra-cellular and cell wall associated products. When target products are cell associated the initial fractionation processes becomes more challenging. Prior to harvesting, cells are weakened through the addition of detergents or other agents so as to release the product from the cell wall or completely lysed or homogenized to release the desired product from within the cells. The consequence of this mode of processing is the resultant release of the cell's entire contents into the process batch.

When evaluating cell lysates, knowledge of the particle size distribution is very useful. Many times the particle size is empirically 'determined' by running several trial and error filtration tests on the harvest solutions. Since the target product is now mixed with the cell broth, each separation becomes somewhat unique.

Separation of disrupted cell masses requires detailed investigation. Successful harvesting of the target product may involve the use of crossflow filtration in combination with other technologies forming a process train also including centrifugation and lenticular and other cartridge filters.

In instances where centrifugation is used as the primary separation step, crossflow is very effective and may offer an economic advantage over other competing technologies especially very large scales of operations.

Ultrafilters

Concentration

The yield equation for concentration is given by:

$$Y = \left(\frac{V_o}{V_f}\right)^{R-1} \tag{5}$$

where

Y = the % yield,
V_o = the starting volume,
V_f = the final volume,
R = the retention coefficient.

The flux response for most low fouling or non fouling crossflow filters is as shown previously in Equation (2). That is the flux declines in a semi log fashion with concentration factor or percent protein in solution (Fig. 6).

Diafiltration

The yield equation for diafiltration on an ultrafilter is given by:

$$Y = e^{\left[\left(\frac{V_d}{V_o}\right)(R-1)\right]} \tag{6}$$

where

Y = is the % yield,
V_d = is the volume of diafiltrate buffer,

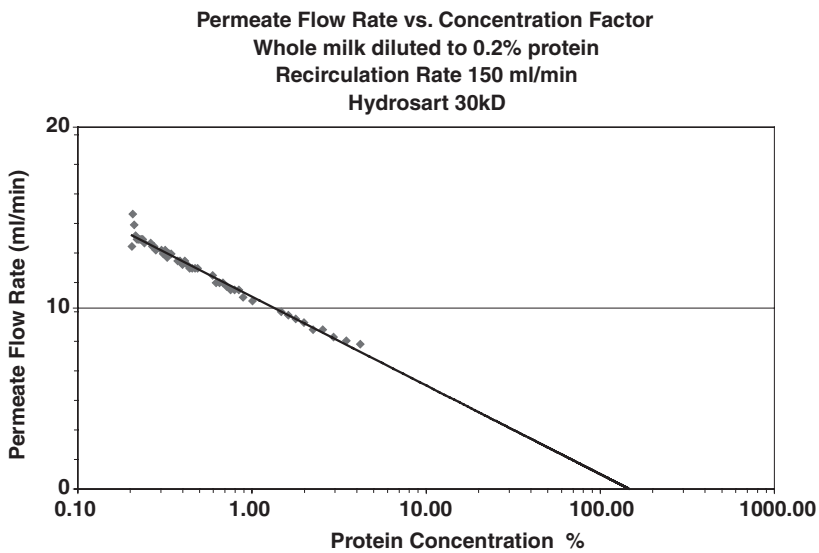


FIGURE 6 Permeate flow rate vs. % of protein on a Sartorius 200 cm^2 30kDa Hydrosart cassette.

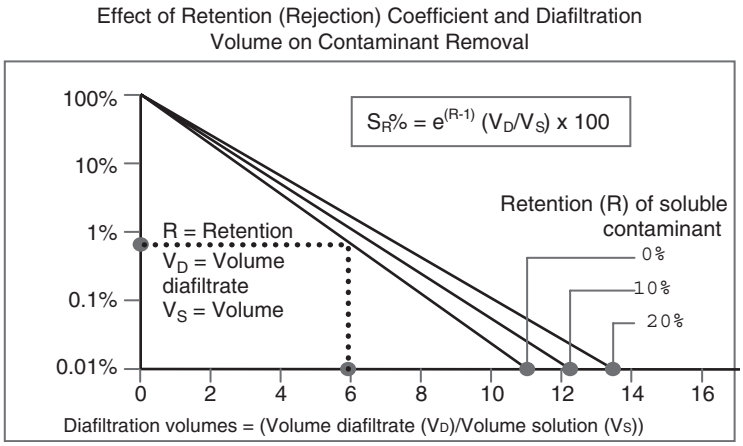


FIGURE 7 Theoretical contaminant dilution versus diafiltration volume.

V_o = is the starting volume
R = is the rejection coefficient.

Thus, if the rejection coefficient is high so will be the yield when utilizing diafiltration. In effect, a percent of the product to be retained is actually passing through the membrane in accordance with the equation above and lost to drain.

This relationship is exactly that utilized in crossflow MF processing as well. In both MF an UF processing the volume of diafiltration buffer required is empirically determined due to potential interactions between the fluid bulk characteristics and the desired product or solute passing through the membrane. The theoretical presentation of this process is shown in Figure 7:

Fractionation

By knowing the rejection coefficient of the target molecules and that of the process contaminants one can exploit the differences between them. This may be accomplished through the use of Equations (6) and (7) from the previous section.

Through concentration and diafiltration, one can fractionate a process stream if there is adequate difference between the rejection coefficients of the molecules. For this to work, there must be a large enough difference between the rejection coefficients, typically $>20\%$ (Table 1).

Example (Eycamp, 1996): if one selects a solution which contains 2 proteins, P1, and P2, and they have starting concentration of 1g/L and retention coefficients of $R1 = 0.99$ and $R2 = 0.80$ we can concentrate the product in the first step 10 fold. The result is given in Tables 2 and 3. In this first step the target protein is enriched by a factor of 6.7:1.

In the second step the product is diafiltered against 10 volumes yielding further enrichment of the target (10.4:1)

MODERN APPLICATION HISTORY AND MEMBRANE POLYMER DEVELOPMENT

In the 1970–1980s Ultrafiltration Bio/Pharma applications were primarily limited to the production of vaccines. These solutions were relatively poorly defined and were made up

TABLE 1 The Theoretical Yield Relationships of Concentration and Diafiltration as a Function of the Rejection Coefficient and Permeate Volume

Retention coefficient	Concentration factor	Yield % conc.	Diafiltration volumes	Yield % diafiltration
0.995	10	98.99	10	95.1
	20	98.5	20	90.5
	30	98.3	30	86.1
0.990	10	97.7	10	90.5
	20	97.0	20	81.9
	30	96.7	30	74.1
0.80	10	63.1	10	13.5
	20	54.9	20	1.83
	30	50.6	30	0.248
0.60	10	39.8	10	1.83
	20	30.2	20	0.034
	30	25.7	30	0.000

of whole cell and virus fractions. It was not uncommon to expect considerable loss of flux in these systems. In later years, i.e., 1980–1990 high value IgG's and other biotech products were being developed. The demand for better performing membranes resulted in the introduction of PES, cellulose triacetate and regenerated cellulose filters. Further membrane improvement resulted in improved polyethersulfones and modified regenerate cellulose membranes which featured both excellent performance, and chemical stability.

CHARACTERISTICS OF CROSSFLOW MEMBRANES

Rejection Coefficient

Membranes are designated by their "rejection coefficient" by the various manufactures to delineate different membrane "cut offs" offered for use. Rejection is a determination of the amount of target molecule that is retained by the membrane being tested.

$$R = 1 - \left(\frac{C_p}{C_f}\right) \tag{7}$$

where

R = the rejection coefficient,
C_p = the concentration of the target solute in the permeate,
C_f = the concentration of the target in the feed.

Membrane molecular weight cut off (MWCO) values are published by the manufactures of membranes based on the rejection coefficients determined and presented by the manufacturer. The correlation to actual and reported MWCO is somewhat subjective, as different suppliers may use different markers and different rejection targets.

TABLE 2 Concentration Yield of Proteins P1 & P2

	% Yield. in retentate	Concentration in retentate (g/L)	% in Permeate	Protein ratio
P1	97.7	9.77	2.3	1.5
P2	63.1	6.31	46.9	

TABLE 3 Diafiltration Yield of Proteins P1 & P2 after Concentration

	Yield in retentate	(g) Concentration in retentate (g/L)	% Recovery	Protein ratio
P1	$.905 \times .977 = .884$	$0.905 \times 9.77 = 8.84$	88.4	10.5
P2	$.135 \times .631 = .085$	$0.135 \times 6.31 = 0.85$	8.5	

As a result manufacturers' determinations and designations may not be similar one to the other and can only serve as a guideline for selection. The MWCO of a membrane is determined by using either specific molecular weight marker molecules or the use of mixed dextrans as shown in Figure 8. Moreover, MWCO values are not absolute. It is typical to refer to the MWCO as the point where the membrane (within QC acceptance limits) rejects a percentage of marker molecules based on the manufacturers designated (67–98%) rejection levels. Another feature differentiating membranes is the steepness of the rejection versus marker plot. A membrane's selectivity may be considered greater and better defined when smaller markers have lower rejection coefficients. For example if 2, 100 kDa membranes may have retention coefficients for albumin (67 kDa) of 80% and

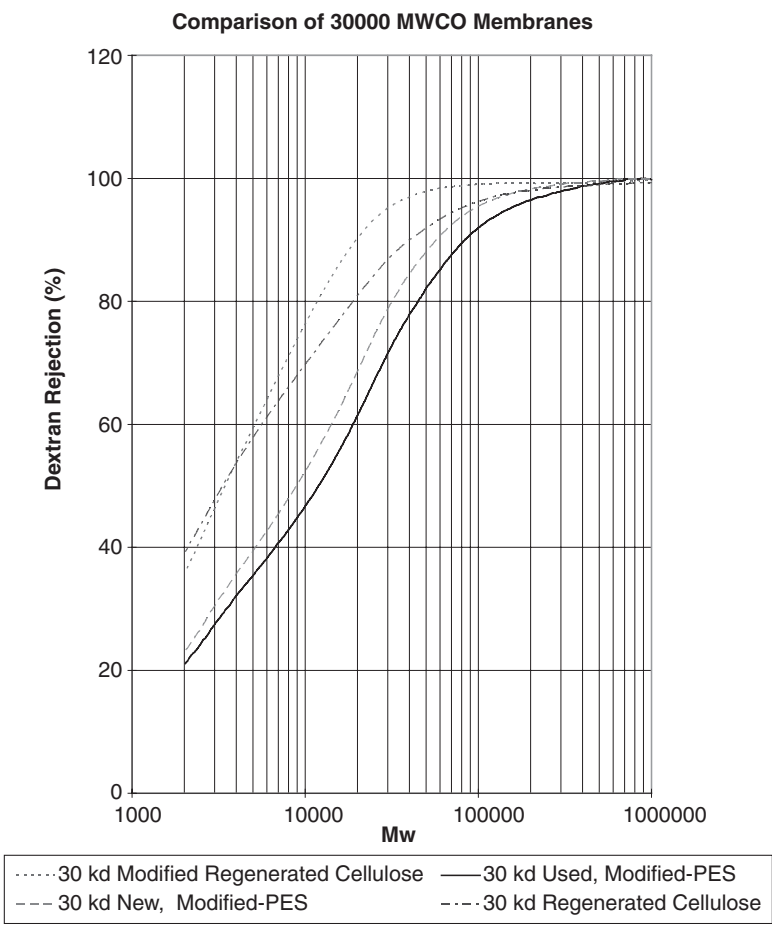


FIGURE 8 Rejection of mixed dextrans on 3 membranes as determined by size exclusion.

40% respectively then one might consider the membrane with a albumin rejection of 40% to have greater selectivity.

By knowing the rejection coefficient of the target molecules and that of the process contaminants one can exploit the differences between them (see fractionation).

Protein Adsorption on UF Membranes

Protein adsorption onto the surface of a membrane is called fouling. Proteins can reacts with, and adhere to, the polymer surface due to hydrophobic/hydrophobic interactions between the membrane and protein. Fouling can also occurs due to charge differences between the polymer and protein causing the protein to adhere to the membrane surface. This problem is especially evident while running concentration or time trials to evaluate membrane for a particular process application with a more hydrophobic membrane as shown in Figure 9.

A truly fouling process does not follow the standard equations. Furthermore, fouled membranes may not be able to be cleaned post-use because the bond between the foulant and the membrane is irreversible. Some reactor antifoams may show such characteristics.

Membrane Fouling

Membrane fouling is a process that results in a marked decrease in performance of Ultrafilter membranes. The fouling of ultrafilters has been inextricably linked to protein processing due in part to the extended history of the membranes that have employed. By understanding the fouling phenomenon we can often minimize or totally eliminate this problem through the proper selection of currently available membranes such as stabilized regenerated cellulose ultrafilters.

A consequence of protein adsorption is a decrease in membrane permeability and an increase in membrane fouling (Marshall et al., 1993; Levy and Shehan, 1991). It has been shown that protein adsorption is greater on hydrophobic membranes than on hydrophilic membranes. Additionally, protein conformation influences membrane performance. Globular or spherical protein deposits influence flux decline less than

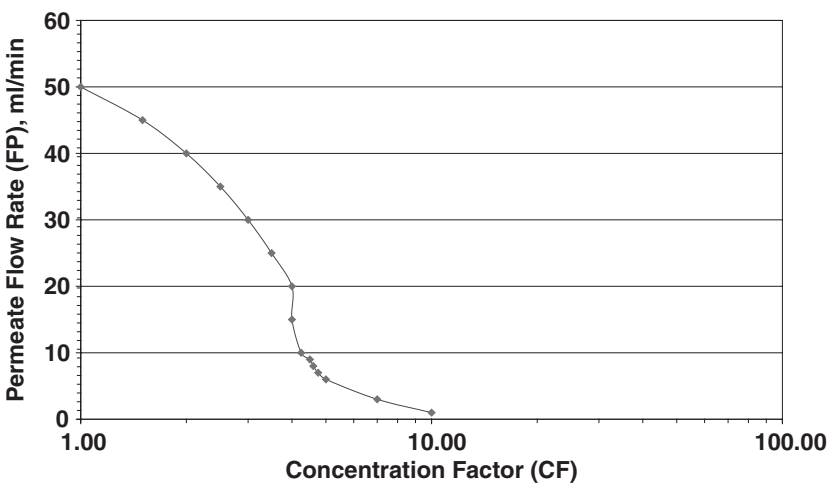


FIGURE 9 Permeate flow rate (FP) vs. concentration faction (CF) fouling stream.

deposition of protein sheets. Freeze-fracture and deep-etching techniques have shown that the deposition of BSA onto hydrophilic regenerated cellulose membranes is spherical in nature. The size of the deposited spheres is consistent with the accepted size of the BSA molecule. However, the deposition of BSA onto hydrophilic polysulfone membranes is filamentous. The conclusion is that the tertiary structure of the protein has been disrupted through the interaction of the solute and the membrane (Marshall et al., 1993).

When fouling occurs the nature of the feed stream composition can have dramatic effects on the retentive nature of the membrane. The retention of large-molecular-weight components can increase the retention of smaller molecular weight components. Blatt et al. (1970) demonstrated that human serum albumin (67,000 Da) retention on a 100,000 MWCO membrane was nearly zero. However when γ-globulin (160,000 Da) was added to the feed stream, the albumin retention rose. Albumin retention showed a linearly increasing correlation to increasing concentrations of the γ-globulin (Blatt et al., 1971). Porter similarly showed that the retention of ovalbumin, chymotrypsin, and cytochrome C are increased when a 1% solution of albumin is added to the feed mixture. The rejection of IgM on 100,000 MWCO Polyethersulfone membranes and stabilized regenerated cellulose membrane show divergently different results even though the mixed dextran rejection profiles are similar. These differences can be attributed to membrane fouling and the nature of the resultant protein cake that forms as described above.

Membrane Types

MF membranes, nanofilters and UF membranes may differ considerably in their morphologies. This can be visualized through SEM scans of cross-sectional cuts of these membrane's. The symmetry of microfilters usually ranges from being uniform to being slightly asymmetric. Many are standard MF filters in crossflow format.

Ultrafilters, on the other hand, are either anisotropic (highly asymmetric) with the rejecting layer consisting of a tight skin (0.5–10 μm thick) supported by a thick spongy structure of a much larger pore size, or "void free" membranes cast onto a MF substructure, with no apparent "skin." Liquid generally flows through the membrane in the direction from the tight "skin side" towards the open side of the UF membrane; flow in the reverse direction can result in the delamination or separation of the UF part of the structure from the substructure of the membrane.

Nanofilters vary in their presentation forms from being composites (tight membranes cast onto a UF or MF membrane) to bi-anisotropic.

The current ultrafilter membrane materials used in the pharma/biotech industry today are, regenerated cellulose, modified regenerated cellulose (Hydrosart™), PES and modified PES. The membranes used in microfilter applications include modified regenerated cellulose (Hydrosart™), PES, cellulose acetate, polypropylene, and polyvynilidine difloride (PVDF).

Photographs 1 and 2 show the two types of Crossflow membrane structure used today. While photographs 3 and 4 show typical types of MF membranes. nanofilters, or virus retention filters are similar in structure to the "void free" UF membranes.

Membrane Polymers

UF membranes are available in a variety of polymers. Some of those polymers have surface modifications which are intended to improve their performance. Today in the pharmaceutical and biotech environment there are two primary polymer families which are in use. These are the polysulfones, and the cellulosics.

The polysulfones include hydrophilic polysulfone and polyethersulfone which represent the polymers that are most widely used due the length of time that these polymers have been on the market. These membranes gained acceptance for use in validated processes in part because of their robustness and the fact that they can withstand 1N NaOH exposure. NaOH is commonly used throughout industry as a means for chemically cleaning, depyrogenating and sanitizing process equipment. Natural polysulfones are hydrophobic. In the casting of these membranes the polymer is treated with hydrophilization agents that render the polymer hydrophilic. Depending on the hydrophilizing agent and process the resulting membrane has a greater or lesser lipophilic profile.

Cellulosic membranes have also been available for many years in the form of cellulose acetate, cellulose nitrate, mixed esters of cellulose, cellulose triacetate, and regenerated cellulose. The short coming of all of these membranes, their chemistries notwithstanding, is their general lack of robustness and limited pH range (4–8). Because of these limitations these membranes have been more difficult to clean in a manner acceptable to the biopharmaceutical industry. In the mid 1990s however, stabilized regenerated cellulose ultrafilters were introduced which overcame the pH limitation and extended the polymers pH range from pH 2 to 14, thus allowing the membrane to be cleaned with up to 1N NaOH. Moreover, some modified regenerated cellulose cassettes are steam sterilizable. Cellulose ester ultrafilters filters are generally in a hollow fiber format, and are often supplied in a single use presterilzed disposable format so as to overcome cleaning and sanitizing issues associated with this type of membrane.

The PVDF membranes include PVDF (hydrophobic) and surface modified (hydrophilic) PVDF supplied in a steamable format. These membranes gained acceptance for use whole cell harvesting primarily because of the open channel device format in which they were offered. Hydrophilic PVDF however is not compatible with 1N NaOH exposure.

Protein-Membrane Chemistry

Membrane polymer chemistry plays a crucial role in the interaction of a product being filtered and the membrane. The polysulfones are rich in conjugated benzene rings which serve as potential sites for hydrophobic/hydrophobic (i.e., membrane–protein) interactions. Likewise, the nitrate groups on the cellulose nitrate and mixed esters of cellulose interact strongly with proteins and other bio-molecules. When such interactions occur the result is the adsorption and denaturation of proteins at the membranes' surface. The effect of the membrane polymer on a protein containing solution has been well documented. Truskey et al. (1987) measured protein adsorption, circular dichroism, and biological activity of variety of protein solutions i.e., insulin, IgG, and alkaline phosphatase, Truskey measured the solution's properties before and after passing the proteins through a variety of membranes. Observed shifts in circular dichroism and decreases in the activity of the enzymes were determined to be the result of conformational changes of the protein structure. This study showed clearly that membranes with the greatest degree of hydrophobicity had the greatest effect on protein adsorption and protein deformation (Truskey et al., 1987). The result of these protein-membrane interactions is that the protein's internal hydrophobic sites become exposed. These exposed hydrophobic surfaces then serve as sites for membrane-protein binding, protein-protein binding, and protein denaturation. This protein-membrane interaction ultimately leads to an overall increased rate of membrane clogging and fouling, reduced performance and loss in product yield.

Data

Figure 10 shows the flux versus TMP profile for Saline, 0.1% lysozyme and 0.1% lysozyme and 0.2% BSA on 30 kDa modified regenerated cellulose membranes. Flux determinations were made at low and high recirculation rates. The profiles for lysozyme/BSA solution and the lysozyme at the low recirculation rate showed the traditional flux limited TMP independent performance resulting from membrane fouling and protein polarization. At the higher recirculation rate the lysozyme flux increased linearly with increasing TMP.

Figures 11 and 12 are the flux versus TMP curves for 10 and 30 kDa membranes before and after protein (1:20 diluted skim milk) concentration and 0.1% lysozyme concentration. For the 10 kDa membranes the modified regenerated cellulose membrane's flux was completely recovered through dilution where the flux on the PES remained irreversibly depressed. After the concentration of the lysozyme flux on 10 kDa modified regenerated cellulose not recovered through dilution.

Figures 10–17 examine saline flux versus. TMP through the different membranes tested. Membranes are tested when new and then again after 2–3 cycle of use followed by cleaning.

Discussion

Post use flux can be completely recovered after protein filtration on modified regenerated cellulose membranes as compared the irreversible and permanent loss in the case of PES membranes. This fundamental difference in membrane performance is due to membrane fouling.

Depending on the membrane foulant PES membranes can loose 75% of its flux where as modified regenerated cellulose membrane flux loss ranges from 0 to 31% loss

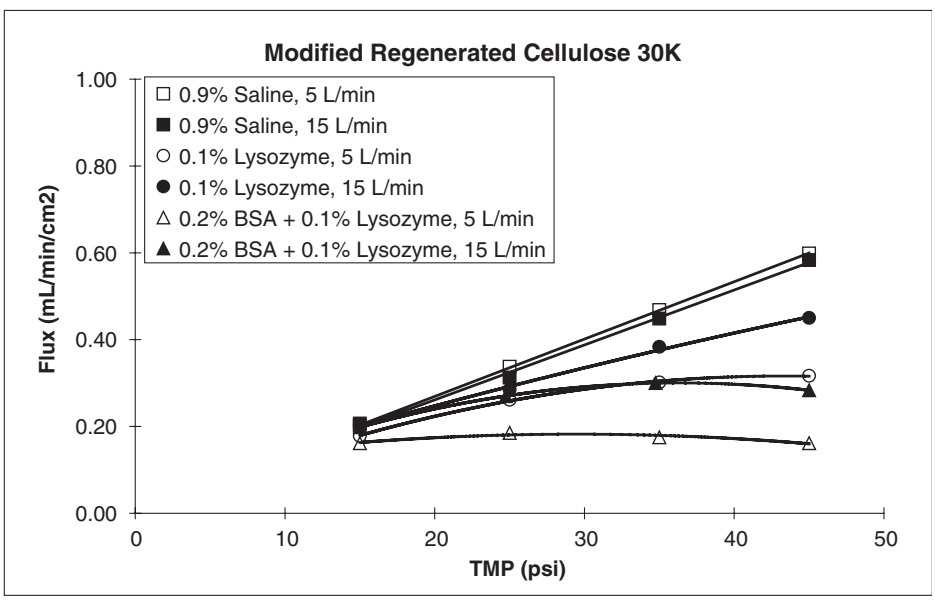


FIGURE 10 Flux vs TMP profiles for saline, lysozyme and lysozyme/BSA solutions at low and high recirculation rates.

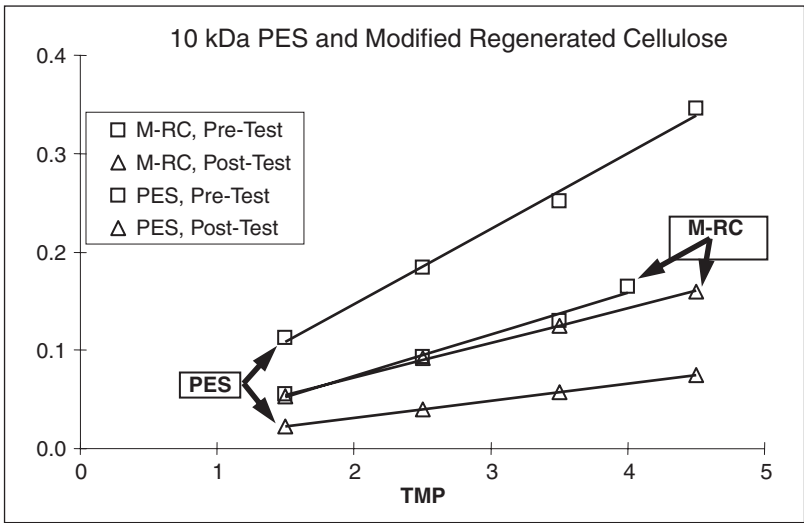


FIGURE 11 Saline flux on 10kDa polyethersulfone (PES) and modified regenerated cellulose membranes before and after the 10X concentration of skim milk diluted 1:20.

after post use rinsing. After cleaning though the modified regenerated cellulose membrane saline flux recovery is usually between 89% and 100% versus the PES membranes where permanent flux loss can be as high as 48%.

Conclusion

Though product rejection and membrane fouling have long been part of the ultrafiltration process, current stabilized regenerated cellulose ultrafilters offer

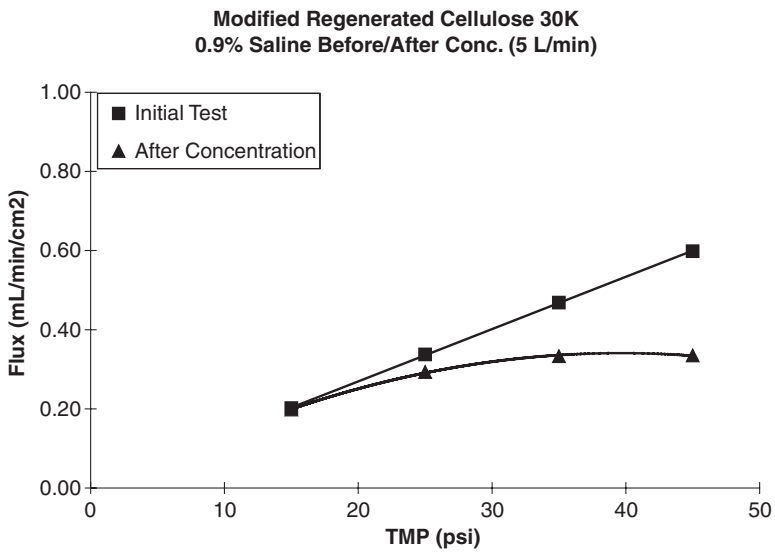


FIGURE 12 Saline flux vs TMP on 30kDa modified regenerated cellulose membranes before and after the 10X concentration of 0.1% lysozyme.

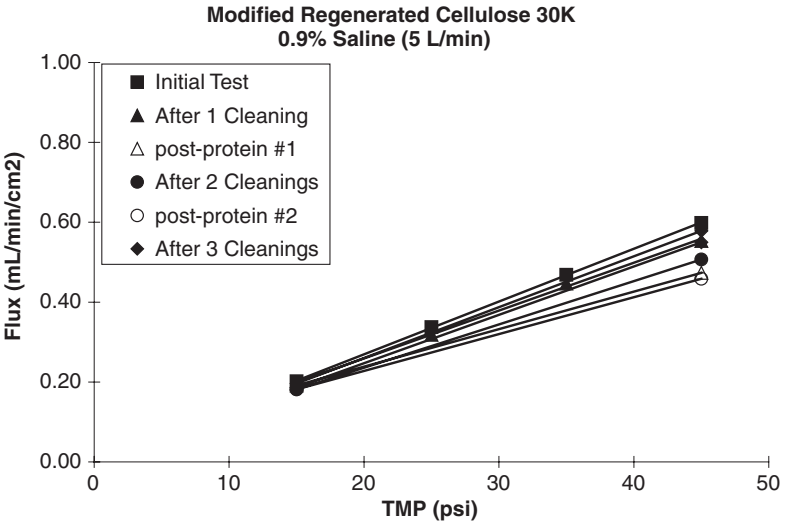


FIGURE 13 Saline flux on 10kDa modified regenerated cellulose (Hydrosart) membranes before and after 3 cycles of 10X concentration of lysozyme followed by cleaning with 1NaOH.

significant performance advantages. The increase in hydrophilic feature in these membranes limits the fouling process. This in turn minimizes the effect of the polarizing protein layer on the membrane surface. Rather than the process forming a "secondary membrane" (protein gel) which eclipses the intrinsic properties of the base membrane the properties of regenerated cellulose membranes are preserved. The result is improved overall performance, easier cleaning and substantially better recovery of flux.

CROSSFLOW ELEMENT DESIGN AND GEOMETRIES

Turbulence-Promoting Insertions

Insertion of static mixers into the retentate flow path enhances the transition of the flow from laminar to turbulent. The use of screens or meshes as static mixers in-between membranes are found in variety of crossflow devices. Screens are used in spiral-wound cartridges and in some plate-and-frame designs. These mesh-like spacers can cause considerable turbulence and have been shown to improve flux (Cheryan and Chiang, 1984). There is some debate as to the nature of the flow through these systems. Belfort (1987) considers the flow to be laminar through systems with screened channels, whereas Cheryan, (1986) reports the flow as turbulent based on the pressure drop within the flow channel. This issue though can be resolved in a straight-forward manner by experimentally determining the slope of log-log plots of flux as a function of velocity as shown in Figure 2.

There are potential down sides to the indiscriminate use of turbulence-promoting insertions for bio/pharmaceutical filtrations. Specifically, some products may be caught on the mesh, creating a cleaning problem as well as causing potential occlusion of the flow channel (Application notes, Sartorius Corporation; Cheryan, 1986). Particulates tend to either hang up on the mesh or occlude the flow channel, so solutions with suspended solids require pre-treatment of the feed. Usually 50-200 μm prefiltration of the feed will

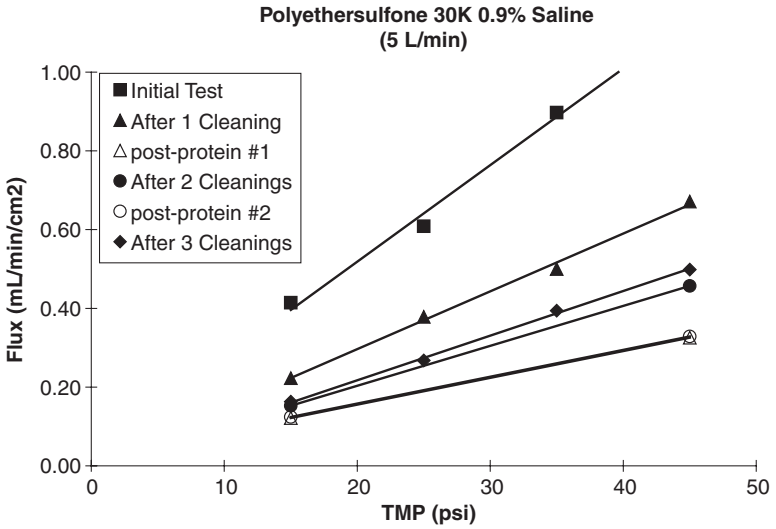


FIGURE 14 Saline flux on 30kDa polyethersulfone membranes before and after 3 cycles of 10X concentration of lysozyme followed by cleaning with 1 NaOH.

alleviate this problem. Meshes are used in construction of cassettes and spirals to keep the feed flow paths open.

Flow-Path Length

The way to control the filter cake is to optimize velocity and turbulence at the membrane surface. The length of the flow path has direct and indirect bearing on these hydraulic

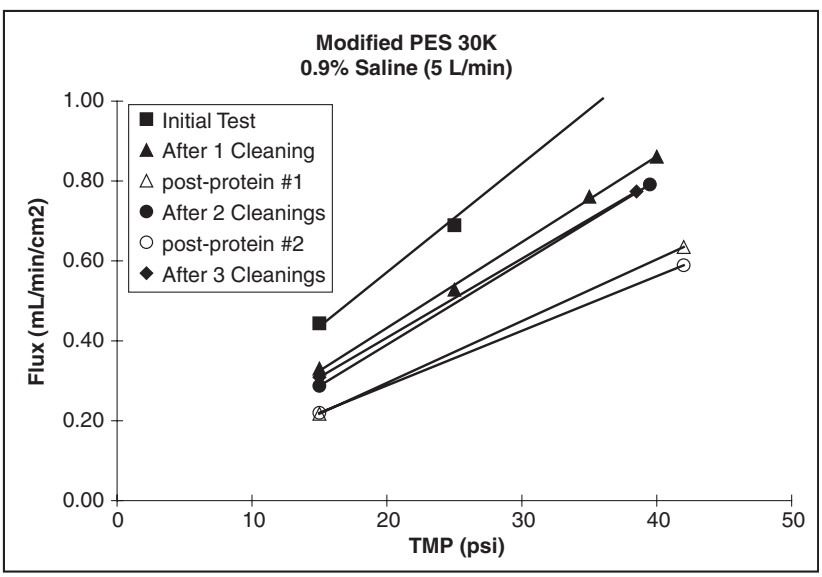


FIGURE 15 Saline flux on 30 kDa modified polyethersulfone membranes before and after cleaning with 1 NaOH.

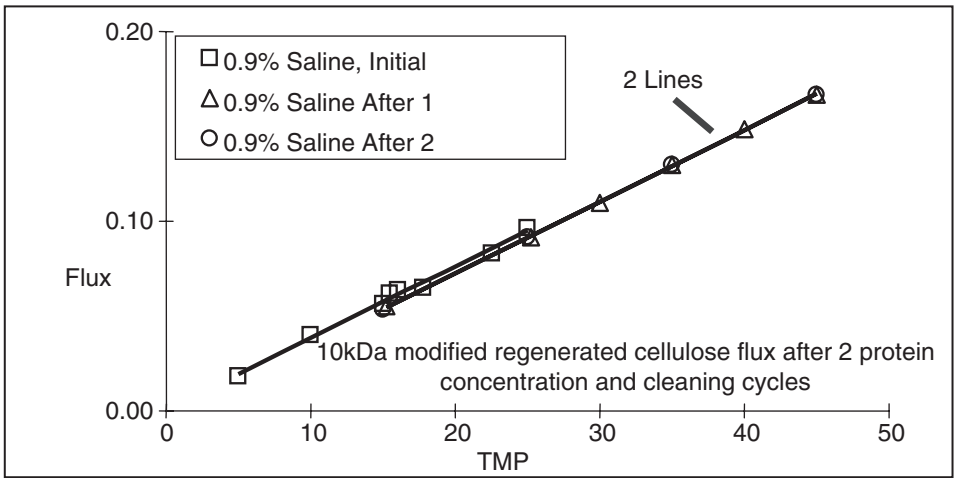


FIGURE 16 Saline flux on 10 kDa modified regenerated cellulose (Hydrosart) membranes before and after 3 cycles of 10X concentration of 1:20 diluted Skim Milk followed by cleaning with 1 NaOH.

forces. First, as shown in Equation (3), when flow is laminar the flux is proportional to the inverse length to the 0.33 power, $(1/L)^{0.33}$. Therefore, increasing the flow-path length has the effect of decreasing flux. In turbulent flow, the length of the flow path does not have a direct bearing on flux. Second, the flow-path length has an indirect bearing on flux for both laminar and turbulent flow because pressure drop through the crossflow device is proportional to the flow-path length caused by frictional forces at the fluid-membrane

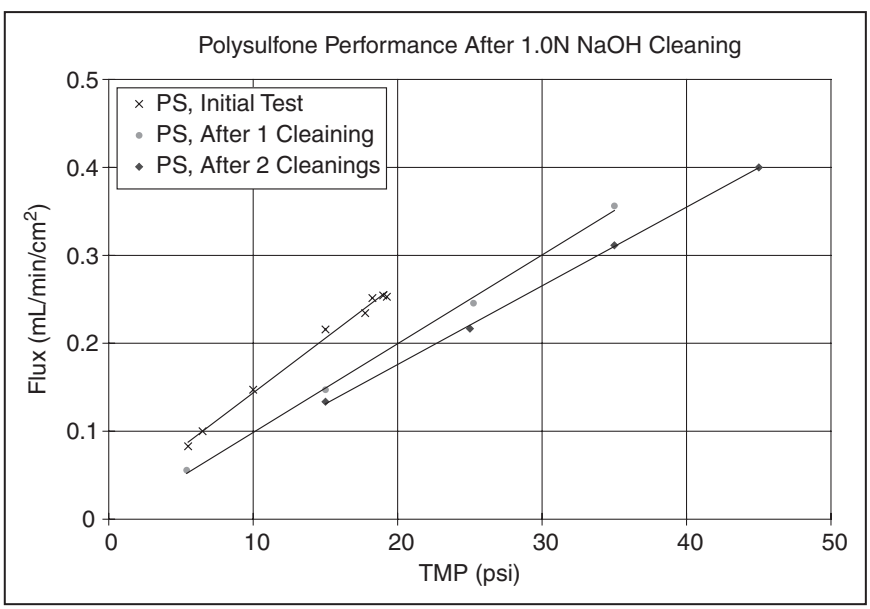


FIGURE 17 Saline flux on 10kDa modified polyethersulfone membranes before and after 3 cycles of 10X concentration of 1:20 diluted skim milk followed by cleaning with 1 NaOH.

interface. Therefore, the longer the flow path the greater the pressure drop and the lower the flux. Third, because fluid is continually permeating the membrane, as the flow-path length increases the volumetric flow and velocity of the feed solution decreases. Equations (3) and (4) show that decreasing velocity causes a reduction in flux. Therefore, based on both direct and indirect reasoning, increasing the length of the flow path causes lower flux. Oddly, the higher conversion of feed to permeate in some membrane devices actually results in a lower overall flux due to the reduced retentate flow if the flow path length is not taken into account during scale down studies.

Flow-Channel Height

The flow-channel height also has direct and indirect bearing on flux in crossflow ultrafiltration. As Equations (3) and (4) show, in laminar flow flux is proportional to the quantity $(1/d\text{h})^{0.33}$ and in turbulent flow flux is proportional to $(1/d\text{h})$. Therefore, as the channel height (or hydraulic diameter) increases the flux will decrease. The indirect consequence of changing channel height is to cause a change in the crossflow velocity, assuming constant volumetric flow rate. That is, the cross-sectional area of the flow channel divided by the volumetric flow rate gives the fluid velocity in the flow channel. By increasing the flow-path channel height, the cross-sectional area is increased, resulting in a decrease in the velocity and subsequently in flux.

When possible the channel height should be as small as possible. However, care must be taken to avoid excessive pressure drops in the flow path and not to select a channel height that might trap recirculating particles or require a pump that might destroy existing particle aggregates in the attempt to achieve sufficient crossflow rates (Devereaux and Hoare, 1985).

Crossflow Module Types

As discussed previously, the ability to create shear and reduce concentration polarization is affected by the inherent design of the membrane module. There are numerous module designs to select from for crossflow filtration. The traditional "process" designs used in the biopharmaceutical industry are cassette (plate-and-frame) modules, spiral-wound modules, hollow-fiber and tubular modules, with rotating-surface modules for bioreactor harvesting.

Cassette Modules

These modules consist of flat-sheet membranes mounted into a frame work Figures 18 and 19. In the assembly of these systems each flow path is made up of two membranes that are facing each other. The upstream flow path must be sealed from the downstream permeate side of the membrane. Stacks of pairs of membranes are layered one on top of the other, and the permeate side of each membrane is supported by a rigid and porous spacer plate. In systems making use of preassembled modules the membrane is generally glued onto the spacer plate. The spacer plate may be smooth or have surface features that give the membrane an uneven surface for turbulence promotion. Flow paths are usually open and may be parallel and or in series. In an alternative design not using rigid plates, the membrane's support is achieved by the dynamic interaction of the membranes above and below the permeate flow path.

Feed-flow paths can be open, but most do use screens. Feed enters at one end of the module through a series of inlet ports and exits the other end of the module through the outlet ports. Permeate flow exits through a series of permeate ports. Preassembled

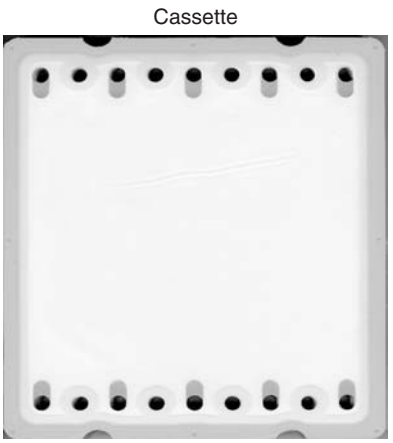


FIGURE 18 Sartocon cassette filter, Sartorius Stedim Biotechs.

cassettes come in a variety of sizes from $50cm^2$ up to $>3m^2$. Systems utilizing these designs may have surface areas as high as 100 m^2 or more (Fig. 20).

The filter holders for these designs are usually constructed from stainless steel, though acrylic and polysulfone versions are available for the laboratory market. The filters are sealed into the holder between two plates via a compression seal. Closure is achieved by tightening tie rods that are attached to the back plate and go through the front plate (Fig. 21). Larger systems achieve compression by using a hydraulics that exerts a force onto a movable front plate, thereby compressing the modules in the middle.

Plate and frame systems are generally easy to clean, have relatively high packing densities, support a wide variety of membrane materials, are amenable to incorporating turbulence-promoting spacers, and provide good crossflow performance. Drawbacks to this design are that fabrication of modules can be labor intensive, and thus expensive; and packing densities are not as great as hollow-fiber modules. However, the advantages to this design outweigh the disadvantages, and this design is widely used in the biopharmaceutical industry.

Spiral-Wound Modules

Spiral-wound modules utilize pairs of flat sheet membranes bounded on the up and downstream sides by screens similar to those in cassette systems. As shown in Figure 22, the membrane sandwich is sealed at three edges so that the feed is isolated

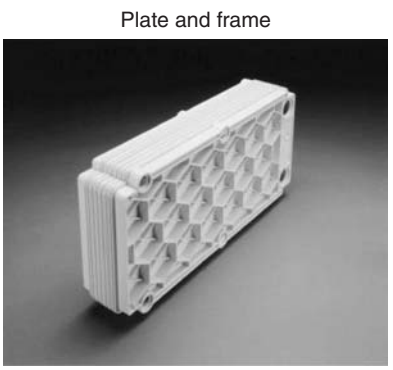


FIGURE 19 Prostack open channel module, Millipore corp.

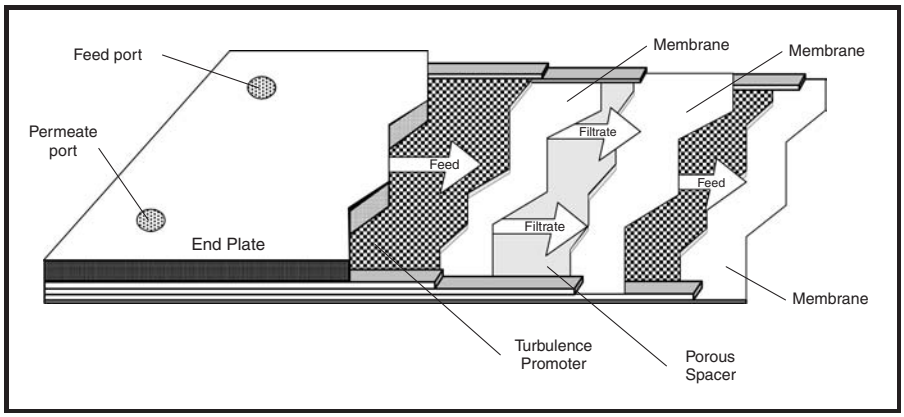


FIGURE 20 Schematic description of flow paths in cassette modules.

from the permeate. The fourth side of the membrane sandwich is attached to a perforated permeate collection tube. The membrane pairs are then rolled around the perforated collection tube, thereby creating the spiral.

Feed flow enters at one end of the spiral, flows tangentially along the axis of the cartridge, and discharges at the other end. Permeate flows at a right angle to the feed flow towards the center of the spiral and is collected in the core of the spiral. Spiral-wound modules are available in a variety of surface areas, starting at 1 up to 50 ft^2. Surface area can be increased by either increasing the number of membrane pairs, increasing the diameter of the spiral wrap, or by increasing the length of the flow path. Spiral cartridges are placed in snug fitting housings. The cartridge is sealed to the housing wall via an o-ring that allows feed flow on the outside of the cartridge. When flow is along the outside of the cartridge it is restricted by a screen so as to provide similar resistance to flow as is seen in the feed flow channels between the membranes. Laboratory devices are available encapsulated in polysulfone housings.

Spiral-wound modules can be ganged together to make filter systems of varying surface areas. Spiral systems feature relatively high membrane packing densities and low

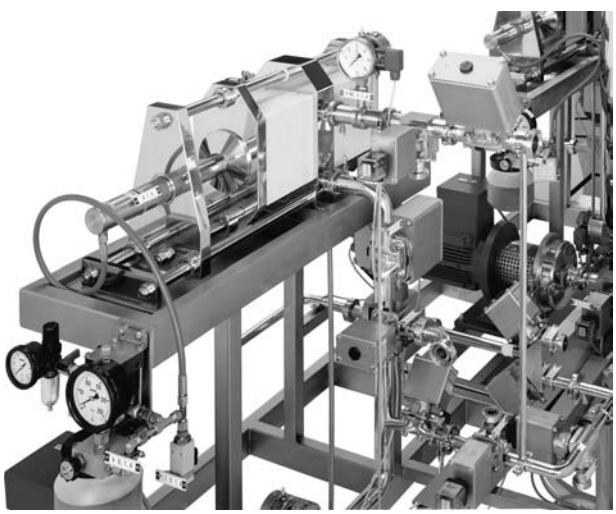


FIGURE 21 Cassette cross-flow modules in a Sartoflow 20 holder (Sartorius corporation).

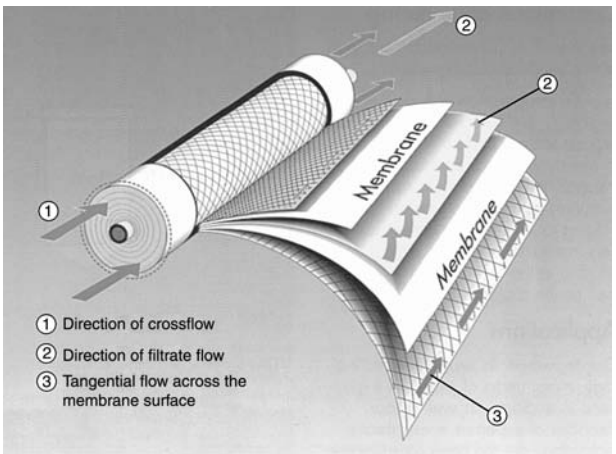


FIGURE 22 Spiral-wound crossflow module.

cost. This design is extremely successful for particulate free process streams but they are difficult to clean when particulates clog the flow paths or collect at the o-ring seals. Other drawbacks to this design include difficulty to sterilize, and long flow paths making high flow with low pressure drop impossible.

Hollow-Fiber and Tubular Modules

The fundamentals of hollow-fiber and tubular crossflow systems are essentially the same, with the difference being that tubes are considerably larger in diameter than are hollow fibers. Figure 23 shows a continuum of "hollow-fiber" modules, with lumen diameters ranging in size from 0.5 to 3.0 mm. Tubular systems may also be configured with removable tubes that are inserted into individual tube holders. In ceramic tubular systems the membrane is cast as part of the ceramic tube array.

The "rejecting" layer of a hollow fiber or tube can be on the inside or outside of the fiber, with the wall of the fiber functioning to support and strengthen the rejecting layer. Liquid permeates the fiber wall, as with flat-sheet membrane, and permeate is collected on the opposite side of the fiber. Hollow fibers and tubes are grouped and sealed into tubular shells to form modules as shown in Figure 23. In the case where the rejecting

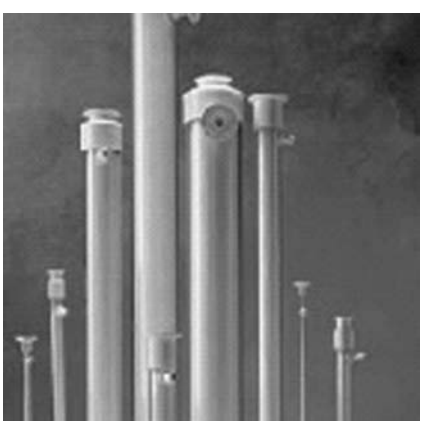


FIGURE 23 Hollow-fiber and tubular modules containing fibers ranging in inner diameter from 0.5 mm to 3.0 mm. *Source*: Photo courtesy of G.E. Health Care.

layer is on the inside (lumen) of the fiber, the feed solution enters the lumen of the fiber at one end, flows down the length of the fiber, and retentate exits at the other end. Permeate is collected on the outside (shell-side) of the fiber.

Hollow-fibers are generally defined as having inner diameter of less than 1 mm. These fibers have greater pressure drop than do tubular modules, however they also have much greater membrane surface area per volume than do tubular modules. Hollow-fiber modules are also less costly than are tubular modules. Ceramic and tubular modules have low packing densities, but because of their large lumen are able to handle very high particle loads. Because of the large lumens they require very high flow rates to maintain high velocity. Ceramic systems have a very high initial installation costs, but because they use inorganic membranes they may have extremely long membrane life.

PROCESS VARIABLES

Filtration Driving Force

Pressure is the driving force for all filtration processes. Plots of solvent flow through a membrane result in a linear increase in the permeate flow as pressure increases. When the solution to be filtered contains solute greater than the filter's pore size then plots of flux versus pressure may become non-linear. When resistance to flow through the membrane increases due to the build up of a solute cake on the membrane then flux fails to increase with increases in pressure.

Trans-Membrane Pressure

TMP is the average pressure on the upstream side of the membrane and is one of the critical controlling factors in the performance of all crossflow filtration applications regardless of the design of the filter module.

$$TMP = \frac{P_{feed} - P_{retentate}}{2} - P_{permeate} \tag{8}$$

Selecting the ideal operating pressures is the result of conducting an experiment where one plots permeate flow rates or flux as a function of the TMP as shown in Figure 24.

There are often three distinct regions when one plots flux versus TMP. In the first phase, flux increases proportionately with increase in pressure. Phase 2 is a transition area and Phase 3 is that portion of the graph where increase in pressure yields no discernable increase in flux. The optimum TMP for a process is generally described as the intersection of the 2 tangent lines drawn through Phases 1 and 3. The benefit of operating at pressures greater than this "optimal" point is off set by the requirement for greater energy input and pumping capacity especially at large scale.

Optimization experiments require that temperature, recirculation rates, and protein concentration be constant. This is because resistance to flow increases as viscosity increases. Viscosity varies directly with concentration and the temperature of the product stream. Resistance to flow is also inversely proportional to the thickness of the solute cake.

One should be cautioned that depending on the available and type of flow measurement instrumentation used, that the recirculation flow rate will be effected as back pressure is applied into the retentate line. Pumps are only able to overcome backpressure to a point after which the recirculation rate begins to decrease. When this happens flux will also begin to decrease in the presence of solute. Figure 25 shows this

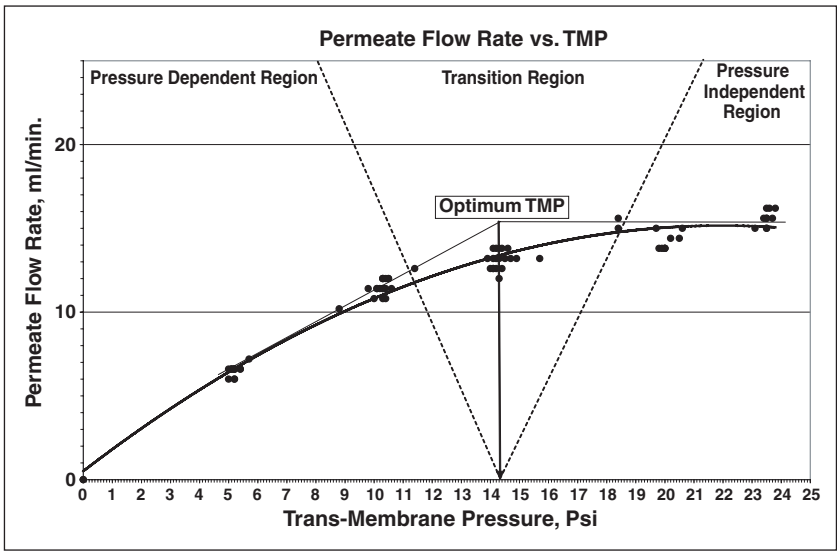

Permeate Flow Rate vs. TMP
Pressure Dependent Region
Transition Region
Pressure Independent Region
Optimum TMP
Permeate Flow Rate, ml/min.
0
10
20
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
Trans-Membrane Pressure, Psi

FIGURE 24 Flux v. TMP at 100 ml/min recirculation rate for skim milk diluted to 0.2% protein in saline filtered through a 200 cm^2 30 kDa Hydrosart ultrafilter membrane.

effect where the pressure drop decreases as backpressure is applied into the retentate line. This loss in pressure differential is an indication of the loss in recirculating flow rate, resulting in a concomitant loss in permeate flux.

Factors Influencing Performance

The factors affecting yield of a protein biopharmaceutical in filtration steps are given in Table 4.

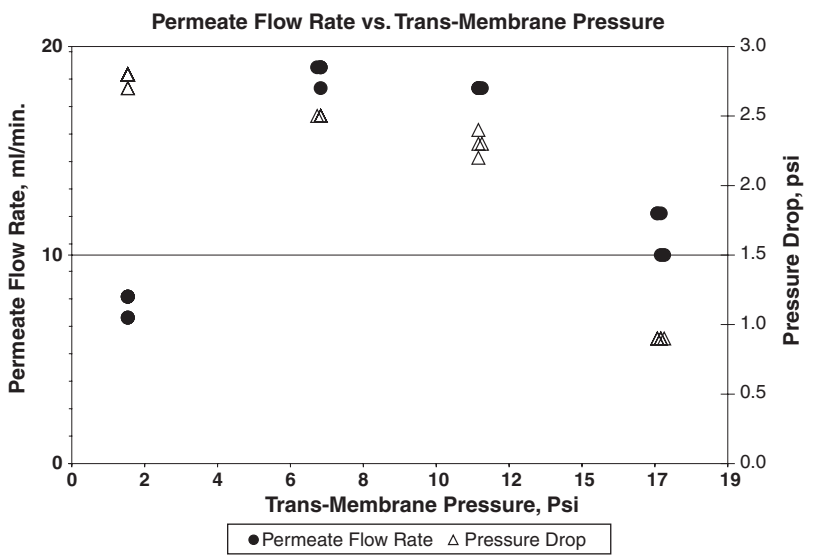


FIGURE 25 Permeate flow rate is plotted versus TMP and the pressure drop between the inlet and retentate with the pump set at 100 ml/min feed on the peristaltic pump for 1:10 diluted milk.

TABLE 4 Factors Affecting Yield of a Protein Biopharmaceutical in Filtration Steps

Process type	Filter type	Points to consider
Cell harvesting	Crossflow microfiltration	Membrane polymer Filter preparation Filter surface area pK_a of the protein Temperature Feed pressure Recirculation rate
Concentration	Crossflow ultrafiltration	Membrane polymer Filter preparation Filter surface area pK_a of the protein Temperature Feed pressure Recirculation rate
Diafiltration	Crossflow ultrafiltration	Membrane polymer Filter preparation Filter surface area pK_a of the protein Temperature Feed pressure Recirculation rate Stability of protein in the buffer system

Feed Stream Composition

The nature of the feed stream can have dramatic effects on the retentive nature of a filtration membrane, especially when the filter is functioning according to the adsorptive-sequestration mechanism. In either mode of filtration, the retention of large-molecular-weight components can increase the retention of smaller-molecular-weight components. Blatt et al. (1970) demonstrated that human serum albumin (67,000 Da) retention on a 100,000 MWCO membrane was nearly zero. However when γ-globulin (160,000 Da) was added to the feed stream, the albumin retention rose. Albumin retention showed a linearly increasing correlation to increasing concentrations of the γ-globulin (Blatt et al., 1971). Porter similarly showed that the retention of ovalbumin, chymotrypsin, and cytochrome C are increased when a 1% solution of albumin is added to the feed mixture. Similar observations have been made regarding the microfiltraton of complex solutions. Most importantly, the actual retention capability of a specific membrane with a specific feed mixture should always be measured experimentally.

Recirculation Flow Rate

Dependence on crossflow rate. Generally, with solutions like buffers and water the permeate flow rate will increase with pressure in a linear fashion. For these solutions, flux is independent of the crossflow rate. Flow through the membrane is only dependent upon the TMP, varying with the fluid temperature.

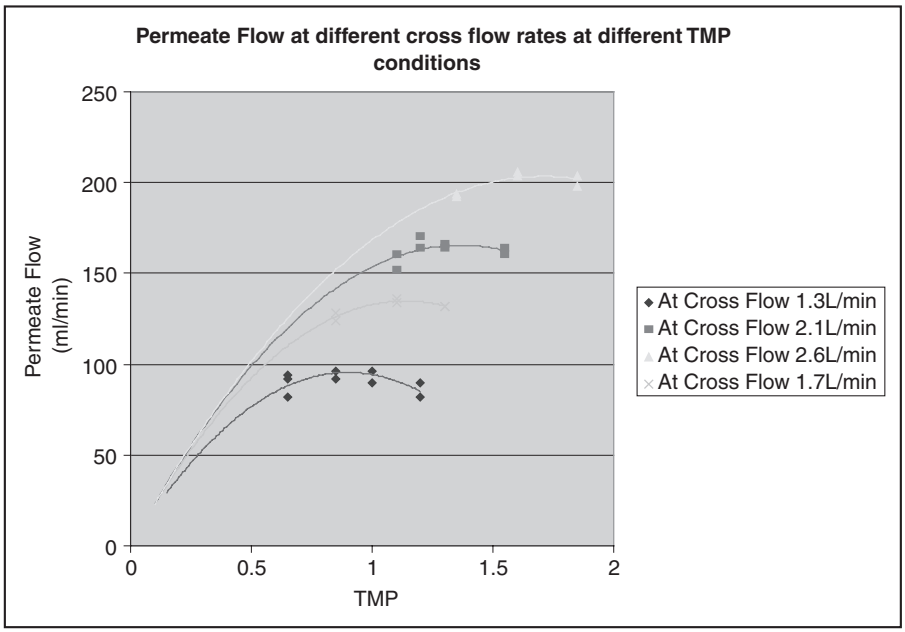


FIGURE 26 Flux vs. TMP at several flow rates and TMP levels.

When processing solutions with retained solutes, flux may be dependent on the crossflow rate. Figure 26 shows a solution where there is a dependence on crossflow rate and the transition of flux from pressure dependence to pressure independence.

Temperature

The effect of temperature on filtrate flux can usually be explained by the changes in solution viscosity. Temperature has a direct effect on viscosity, that is, as temperature is increased viscosity decreases, and as viscosity decreases flux increases. Flux is generally inversely proportional to the viscosity of the feed solution.

Biopolymer polarizing gels that form during filtration of proteinaceous solutions are gelatinous in nature. The plasticity of these components is affected by the temperature of the solution. When the solution is kept cold and viscosity is high, they behave like rigid particles; when they are warmed, they are free to deform from the stresses of the filtration process. Polarized protein cakes are compressible, and the pressure of filtration can cause the (gel) layer to collapse and completely clog the membrane. The temperature of the solution influences the compressibility of the filter cake. When the solution is kept cold (e.g., 4°C) the filter cake is less compressible than when it is warm. As a result, filtration of biopolymer-containing solutions proceeds far better when the solution is kept cold. The increased through-puts when running cold far out weigh the reduction in flux as a result of the increased viscosity of the solution.

Temperature also influences the solubility of certain proteins. Cryoglobulins are a class of serum proteins that precipitate when the temperature of the solution is lowered to a critical limit. Freezing and thawing of protein solutions also results in the generation of protein precipitates. Adding heat to the solution can cause the disruption of hydrogen bonds, causing unfolding of some portions of the protein which in turn results in the formation of aggregates that may precipitate.

If the temperature is raised, protease activity may be activated or accelerated, which will result in protein degradation and precipitation of the proteolytic fragments. Lastly, at higher temperatures the adsorption of protein to the membrane is enhanced–resulting in an increase of membrane fouling.

pH and Ionic Conditions

Protein solubility generally increases as the solution pH moves away from the protein's isoelectric point and at extremes in solution ionic strength. As the isolectric point is approached or as the solution's ionic strength decreases or increases dramatically, the protein will precipitate from solution and likely foul the membrane. Moving the pH too far one way or the other from the isoelectric point charges the protein, causing increased protein-membrane or protein–protein interaction. Under these conditions, proteins may start to agglomerate or bind to the membrane, which will adversely affect the product and the process. Changes in the proteins' conformation and stability affect their adsorption to membrane surfaces. McDonogh (1990) showed that protein fouling is maximized at the protein's isoelectric point and that the phenomenon is concentration dependent (McDonogh et al., 1990).

Buffer exchanges and diafiltrations are common in the processing of protein solutions. It is important to maintain an ionic equilibrium in the solution. When a buffer or salt is exchanged, one must be sure that all of the components remain in solution during the exchange process. Failure to do this may result in protein precipitation and fouling of the system. This is especially true when proteins are being concentrated by UF, because if the salts concentrate with the proteins the proteins may start to salt out. Likewise, if the salt levels falls too far, precipitation may also take place. Calcium and phosphate are directly implicated with the formation of insoluble calcium salts that serve as bridging agents for protein deposits (Marshall et al., 1993;).

Removal of certain ionic species (e.g., by diafiltration) can also result in the formation of precipitates. This happens when the quaternary protein structure is lost, as the result of the removal of cations like magnesium, which serve to bridge the protein subunits. When serum proteins are dialyzed against distilled water, flocculation occurs as a result of the precipitation of a class of proteins called euglobulins which is comprised primarily of IgM.

PROCESS OPTIMIZATION

Optimizing Experiments

The experiments for optimizing an ultrafiltration application are relatively simple and straight forward. They are primarily designed to identify the optimal feed flow rate and TMP.

Clean Water Flux

Clean water flux (CWF) values serve as benchmarks as experiments are conducted. When flux cannot be recovered through dilution we may conclude that fouling has occurred. Fouling can be described as an irreversible process whereby the product stream components become irreversibly bound to the membrane. With proteins, fouling is manifested by the formation of a cake of denatured protein bound to the membrane surface (Dosmar, 2005). When membranes foul, flux may only be recovered after the

membranes are cleaned with NaOH or other aggressive cleaning agents. Depending on the membrane polymer CWF may exhibit a continuous decline following each cycle of use and cleaning.

CWF can be determined by measuring flux at a set temperature and pressure while deadheading the filter. If the water temperature varies then one can normalize the value (Table 5).

$$\mathrm{NCWF} = \mathrm{CWF}^{*}\mathrm{K} \tag{9}$$

where

NCWF = Normalized clean water flux at 25°C
CWF = clean water flux at temperature X
K = correction factor for temperature X

Optimizing the Pressures

Selecting the ideal operating pressures is the result of conducting experiments where one plots permeate flow rates or flux as a function of the TMP (see section on "Trans-Membrane Pressure") and as shown in Figure 13.

As discussed earlier there are generally three distinct regions when one plots Flux vs. TMP (Fig. 24). In the first phase, flux increases proportionately with increases in pressure. Phase 2 is a transition area and Phase 3 is that portion of the graph where increase in pressure yield no discernable increase in flux. The optimum TMP for a process is generally described as the intersection of the 2 tangent lines drawn through Phases 1 and 3. The benefit of operating at pressures greater than this "optimal" point is off set by the requirement for greater energy input and pumping capacity especially at large scale.

These experiments require that temperature, recirculation rates, and protein concentration be constant. This is because resistance to flow increases as viscosity increases. Viscosity varies directly with concentration and the temperature of the product stream. Resistance to flow is also inversely proportional to the thickness of the solute cake.

One should be cautioned that depending on the available and type of flow measurement instrumentation used, that the recirculation flow rate will be affected as back pressure is applied into the retentate line. Pumps are only able to overcome backpressure to a point after which the recirculation rate begins to decrease. When this happens flux will also begin to decrease in the presence of solute. Figure 25 shows this effect where the pressure drop decreases as backpressure is applied into the retentate line. This loss in pressure differential is an indication of the loss in recirculating flow rate, resulting in a concomitant loss in permeate flux.

TABLE 5 Normalized Clean Water Flux: Temperature Correction Factors

Temperature °C	Correction factor	Temperature in °C	Correction factor
25	1.000	19	1.152
24	1.023	18	1.181
23	1.047	17	1.212
22	1.072	16	1.243
21	1.098	15	1.276
20	1.125	14	1.310

Excessive TMP pressure can cause the membrane's substructure to compress or collapse resulting in a progressive reduction in the membrane's flux due to the occlusion of the exit pathways through the substructure.

In Figure 27 membrane hysterisis is observed in 1 and 5 kDa PES ultrafilters. The 1 kDa ultrafilter displays nearly a 40% drop in flux under the influence of a pressure of 4 bar. Under the same conditions the 5 kDa PES membrane looses 10% of its flux. Though this example may be an extreme is does serve to illustrate and alert users that the phenomenon can occur. It should be noted that this phenomenon is less likely to be seen with membranes that have high permeation rates as exemplified by the difference between the 1 and the 5 kDa membranes. Furthermore, the nature of the substructure (foam versus finger) will have a significant influence on whether this phenomenon will occur at all. Membranes with a foam or supported substructure will not lose flux due to compaction.

Flux versus Recirculation Rate

Membrane flux is affected by the thickness of the polarized filter cake. The thickness of that cake may be controlled hydraulically. Increasing the fluid flow rate through the flow path results in an increase in the shear forces acting on the filter cake. The shear forces at any given flow rate vary with the viscosity of the product. By plotting the relationship between the permeate flux vs. TMP at different feed flow rates one can assess the "cost versus benefit" of increasing the recirculation rate. For water like products, the fluid stream will generate greater shear than for a viscous product.

Figures 27 and 28 show that as the recirculation rate increases flux likewise increases at the same TMP.

Preparing log–log plots of flux versus velocity, shear or recirculation rate may provide a view as to the effect of increased recirculation.

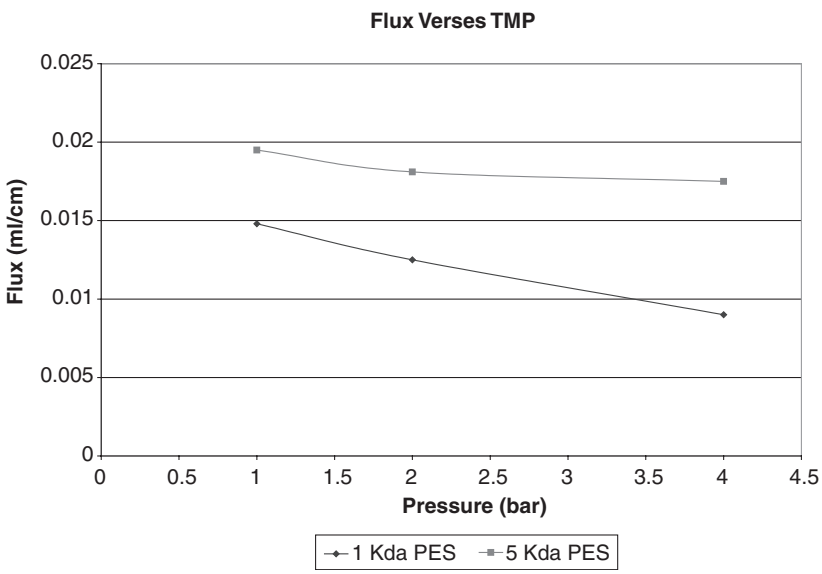


FIGURE 27 The effect of pressure on 1 and 5 kDa PES ultrafilters substructure.

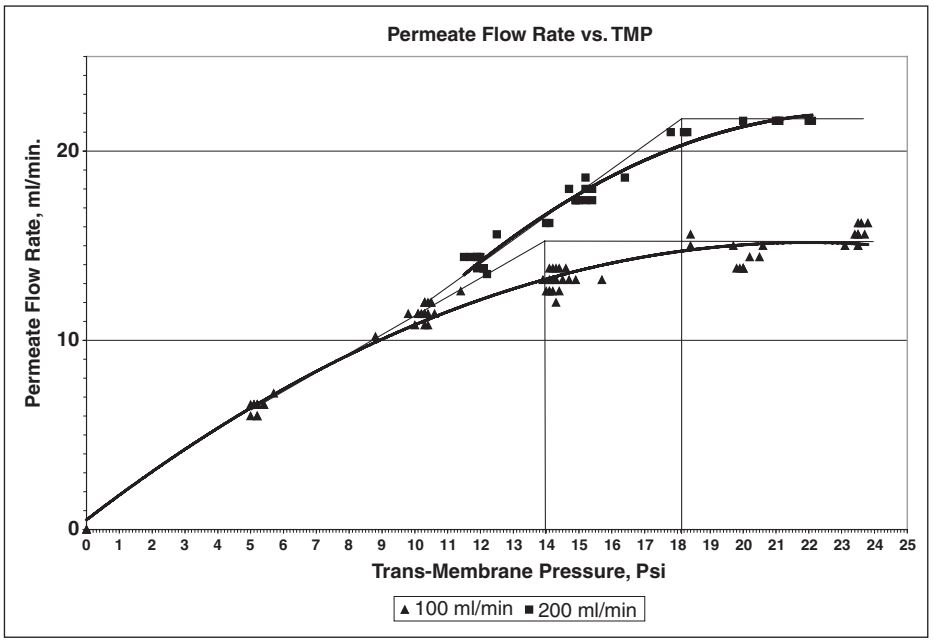

Permeate Flow Rate vs. TMP
Permeate Flow Rate, ml/min.
0
10
20
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
Trans-Membrane Pressure, Psi
▲ 100 ml/min ■ 200 ml/min

FIGURE 28 Permeate flow rate versus TMP at 100 ml/min and 200 ml/min recirculation rate for skim milk diluted to 0.2% protein in saline filtered through a 200 cm^2 30 kDa Hydrosart ultrafilter membrane.

Figure 29 (Dosmar, 2006): shows the permeate flow rate versus Recirculation rate at a TMP of 23 PSI with skim milk diluted to 0.4% protein in saline filtered through 200 cm^2: 2, 10, 30, and 100 kDa Hydrosart ultrafilter membranes.

Figure 28 shows the effect of the membrane pore size on permeate flux and on the slope of the increase in flux with increases in recirculation rate.

If one is working with filters of differing geometries, then plotting the data using flux and velocity may be more useful because it normalizes the results. Velocity can be calculated by dividing the volumetric flow by the hydraulic diameter.

$$V = \frac{\frac{Q}{t}}{d} \tag{10}$$

Q/t = flow rate
D = hydraulic diameter
and

$$D = 4 \cdot \left(\frac{Ch \cdot Cw}{2Ch + 2Cw} \right) \tag{11}$$

where

Ch = channel height
Cw = channel width

For cassettes the total hydraulic diameter is the hydraulic diameter for one channel multiplied by the total number of flow channels in the cassette.

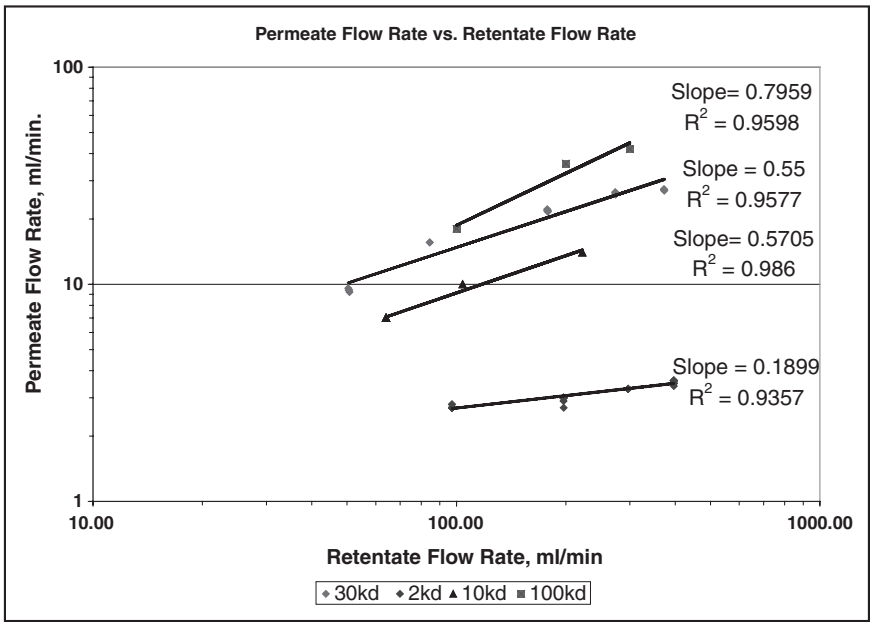


FIGURE 29 Permeate flow rate versus recirculation rate at a TMP of 23 PSI with skim milk diluted to 0.4% protein in saline filtered through 200 cm^2: 2 kDa, 10 kDa, 30 kDa, and 100 kDa Hydrosart ultrafilter membranes.

FLUX VERSUS PROTEIN CONCENTRATION

Plotting flux versus concentration at a fixed recirculation rate, fixed TMP and fixed temperature will allow one to determine where the optimal diafiltration point might be, the required diafiltration buffer volume and the total time required for the process (Fig. 30).

Extrapolating the flow decay line to where the plotted line intercepts the *X*-axis provides a theoretical maximum solute concentration value.

Plotting runs conducted at multiple recirculation rates provides evidence as to whether the solute is fouling or not (Fig. 31).

Non-convergence of flux decay plot to a common end point provides evidence that no protein fouling is occurring. When concentration dependent flux decay plots converge to a common end point regardless of the recirculation rate, one can conclude that the protein solute is forming a fouling protein gel.

PROCESS SCALING

A successful scale-up from small clinical (Phases 1–2) scale volumes to pilot (Phase 3) or commercial production scale is recommended by regulatory bodies, that is, EMEA/The European Agency for the Evaluation of Medical Products (2001). In the "Note for Guidance on Process Validation" section 3.1 "It is expected that during the development stage, the manufacturer of the product should gain sufficient information about the behavior and the physical and chemical properties of the drug substance, the composition

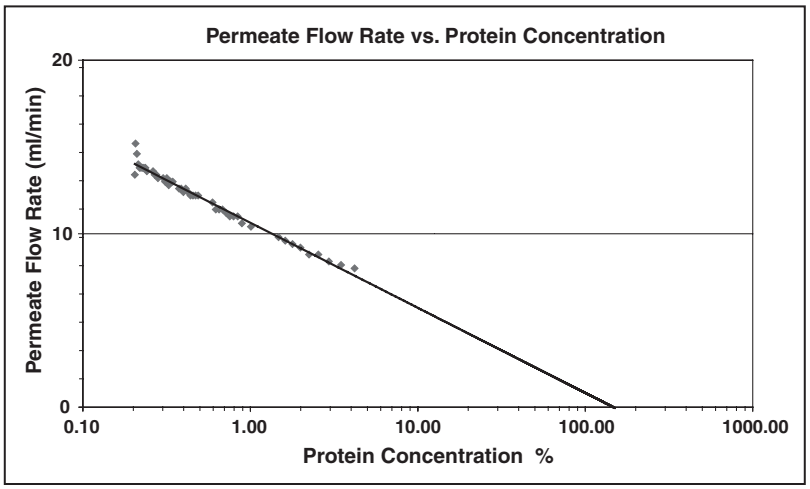


FIGURE 30 Permeate flow rate versus % Protein: Whole milk diluted to 0.2% protein in saline and then concentrated using a Sartorius 200 cm^2 30 kDa Hydrosart membrane at a recirculation rate of 150 ml/min at a TMP of 18 psi.

of the product in terms of active ingredient(s) and key excipients, and the manufacturing process clearly define the critical steps in the manufacturing process."

Small scale experiments conducted in the laboratory, generate data that includes equipment, that is, elbows, valves, and pumps that may skew performance expectations because of the inherent nature of the systems' plumbing. Though one may wish to minimize these effects, they cannot be disregarded.

Elucidating a process allows decision making based on selecting optimal parameters. Experiments looking at flux versus TMP, log flux versus log recirculation

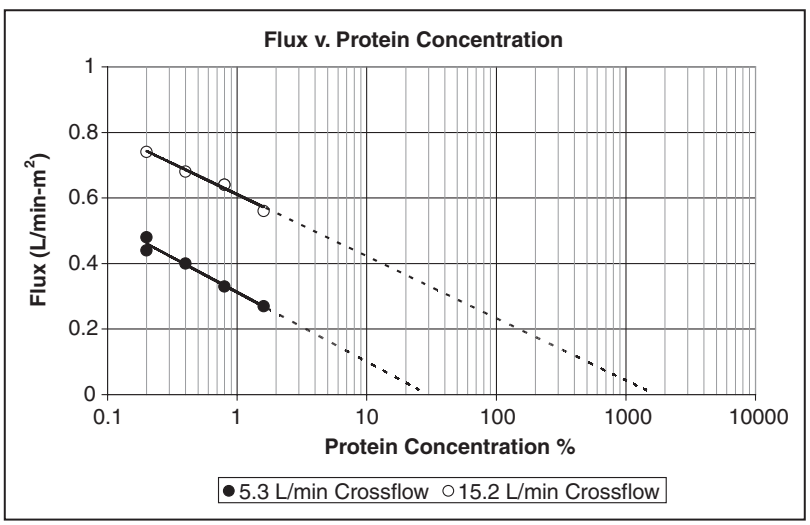


FIGURE 31 Flux vs. protein concentration at 2 different recirculation rates for skim milk diluted to 0.2% protein. Concentration decay plots are extrapolated to where flux approaches 0. (Sartorius Hydrosart 30kDa membrane area = 6,000 cm^2).

rate, identifying the optimal pressures and flows for the process (Dosmar and Scholz, 1995; Brose et al., 1996; DePalma, 2004) must be conducted. These experiments also provide the reference values for the scaling process.

System Hydraulics

Scaling up process piping is typically the result of determining the desired flow rate, pressure and fluid flow velocity. Elbows, valves, and other process components within the fluids path have calculatable effects on both pressure and flow rate. Scaling-up a crossflow system, on the other hand, offers an additional hurdle, that of the hydrodynamics of the fluid flow through a cassette's feed and retentate screened flow paths (Wolber et al., 1988). There are two components to a cassette's feed and retentate flow path. Those are the feed and retentate flow manifolds internal to the cassettes, which feed and receive flow from the membrane flow channels and the flow channel itself. Crossflow cassettes have screens interleaved between each membrane pair on both the feed and permeate flow channels (Fig. 32).

These screens serve as a static mixer. Depending on the screens' designation, the gap between the membranes can vary, and with it the pressure drop through that channel. As part of the cassette's construction, the screens and membranes make up the wall of the manifold feeding the membrane feed channel as in Figure 31. This internal manifold is made up of the edges of the membranes and interleaving screens, therefore it is not smooth like process tubing. As a result the manifold's wall increase resistance to flow because of wall drag to a much greater extent than that for process tubing. Flow rate through the feed manifold increases as one adds surface area (cassettes) because recirculating flow rate per square meter of membrane is generally kept constant as the process is scaled up. The drag at the edges of the manifold can cause the feed solution to transition from laminar to turbulent flow especially at the proximal cassette(s). As this happens pressure drop in this channel develops (Figs. 33 and 34).

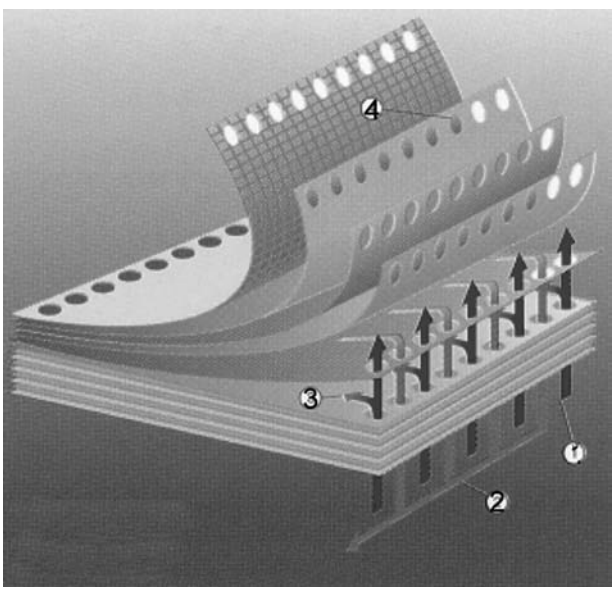


FIGURE 32 Diagram of a cassette where (**1**) is the feed flow through the feed ports of the cassette, (**2**) is the retentate flow, (**3**) is the feed flow through the membrane flow channel, and (**4**) is the membrane.

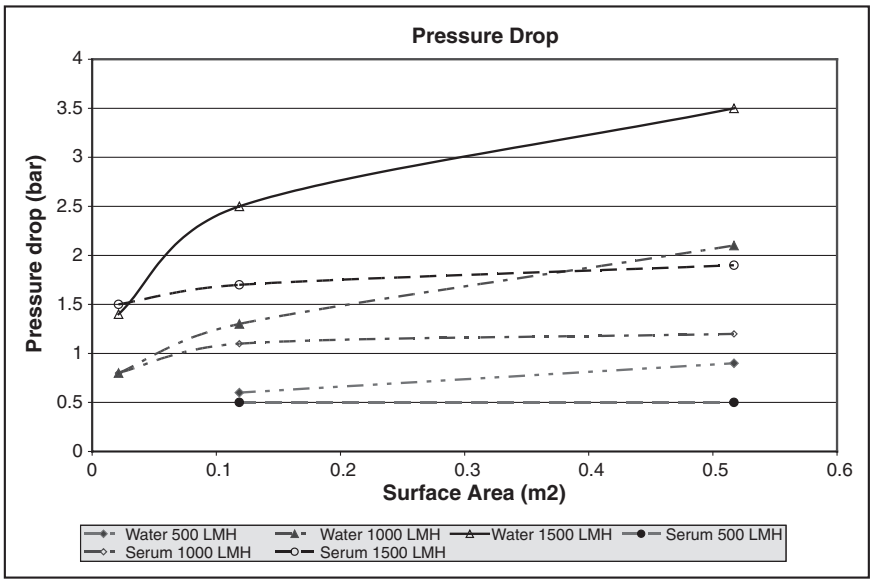


FIGURE 33 Observed pressure drop with water and bovine serum using Sartocon Slice 200 (200 cm^2), Sartocon Slice (1000 cm^2), and Sartocon cassette (5800 cm^2) at recirculation rates of 500, 1000, and 1500 LMH.

Pressure drop is directly tied to a fluid's viscosity in the flow path. Figures 30 and 31 show the effect of viscosity on pressure drop. Here, pressure drop is measured with water and with bovine serum and then plotted against surface area. Regardless of the TMP, and inlet and outlet pressures, the pressure differentials remain constant (data not

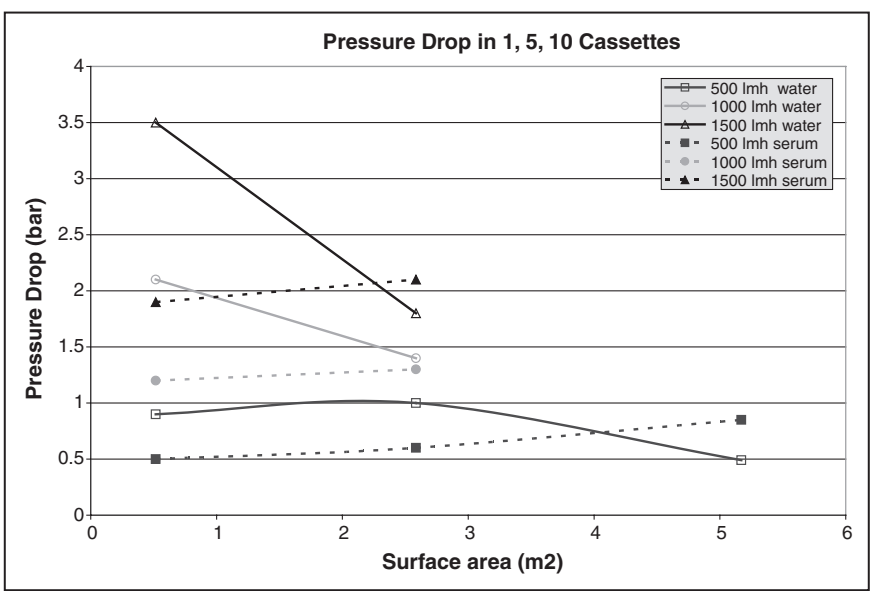


FIGURE 34 Pressure drop in 1, 5, 10 cassettes with water and serum.

shown) at any one flow rate. Reynolds numbers increase as viscosity decreases. Pressure drop likewise increases as the flow through the manifold transitions into turbulent flow. This transition occurs at lower flow rates when the viscosity is low. Cherian (1986) shows the general relationship of pressure drop (ΔP) being directly proportional to flow rate (Q). This relationship is used to determine whether flow rate is laminar or turbulent flow using Equation (12) where for values of $n = 1$, flow is laminar and where values of $n > 1.4$, flow becomes turbulent.

$$\Delta \propto f(Q)^n \tag{12}$$

As surface area is added the pressure required to maintain a constant recirculation rate per square meter of membrane must increase. This increase may not be linear and can vary as a function of the screen designation and flow channel gap width. In short, each membrane flow path creates a resistance to flow. As flow paths are added additional pressure is required to overcome the additive effect of the pressure drops.

Turbulence Induced Pressure Drop

The pressure drop data shown in Figure 31 implies that with water a significant pressure drop occurs at high flow rates as a result of turbulence. This turbulence occurs at the proximal entrance into the first cassette's feed port where there are two "elbow" effects created by the change in the direction of the fluid path. Flow enters the holder and exits at a 90° angle into the cassette. A part of the flow then changes direction a second time entering into the membrane flow channel. Depending on the rate of flow and the abrupt changes in flow direction a region of turbulence can be created when the flow rate is sufficiently high. As cassettes are added, the effect is eliminated because the solution is able to transition back into laminar flow. As more cassettes are added additional flow is required to maintain a constant flow per cassette and turbulent flow can be once again reinitiated if the flow rate is sufficiently high (Fig. 35).

The flow rate for small surface area cassettes $< 200\,cm^2$ relative to both the inflection distances and the port size, assures smooth flow throughout, resulting in no turbulence induced drop in pressure. At the $1000\,cm^2$ size turbulence induced pressure drops can be inferred at high recirculation rates.

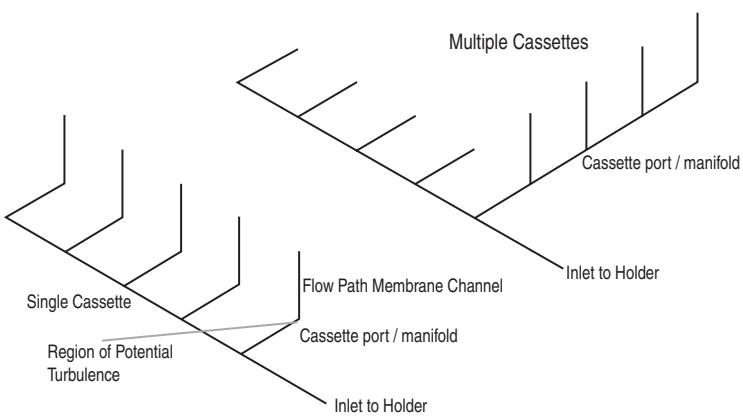


FIGURE 35 Diagram of flow path into a filter holder and cassettes.

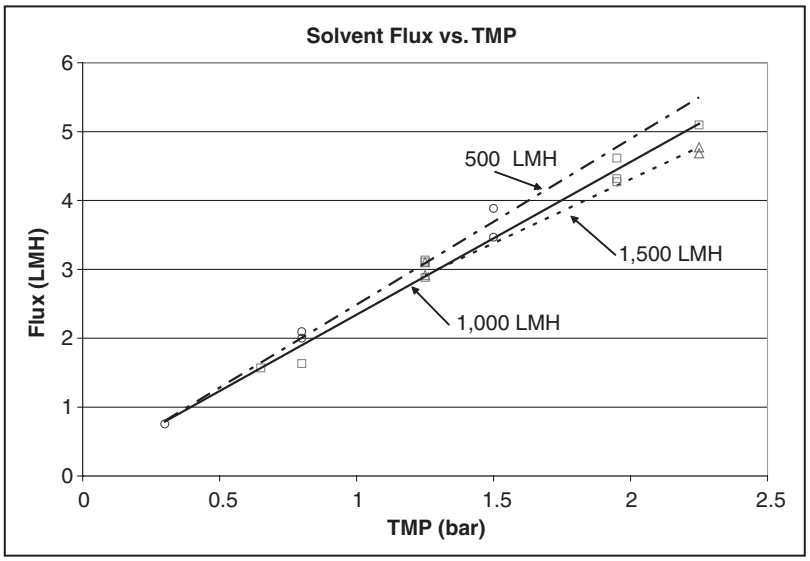


FIGURE 36 Solvent flux (water) is measured at recirculation flux values of 500, 1,000, and 1,500 LMH on a Slice 200 (200 cm^2), Slice (1,180 cm^2) and on 1,5, 10 and 20 Sartocon cassettes (ea 5,800 cm^2) all values are plotted together making no distinction between the devices and relative surface areas. Trend lines are automatically drawn by Excel™ through the data sets.

Since turbulence and viscosity are inextricably linked one can see that the pressure drops seen with water are not observed with product. Depending on the nature of a product's viscosity at the start, middle and end of a concentration run, turbulence induced pressure drops should be accounted for and controlled.

Pressure Drop Induced Flux Loss

When turbulence induced pressure drops occur, the consequence is a drop in permeate flux due to loss of motive force (Fig. 36.). At low recirculation flow rates, solvent flow is greater than at higher recirculation flow rates at the same calculated TMP. This is due to the pressure drop in the feed ports. When the pressure drop occurs prior to entering the membrane flow channel, the actual pressure available for permeation is reduced even though the apparent TMP's are the same (Figure 33).

Surface Area

Surface area is the amount of effective membrane in a system. Commercially available "scalable" cassettes are available in surface areas from about 50 cm^2 to > 3 m^2. Cassettes from a variety of manufactures all claim to maintain common geometries within their respective product lines making them suitable for scale up and scale down studies.

Sources for Variation in Flux

Surface Area

Membrane surface areas reported by most manufactures of UF are in whole number increments. This being the case, rounding errors can contribute considerably to errors in scaling.

TABLE 6 Cassette Scale-Up Factors Determined Experimentally Using Published Values and QC Lot Release Data

Filter / Cassette (actual surface area)	Mean Scale-up factor based on experimental water flux	Scale-up factor based on published area	Scale-up factor based on experimental serum flux	Scale-up factor based on QC water flux	Scale-up based on actual surface area
Slice 200 (180cm^2)	1	1	1	1	1
Slice (1,170 cm^2)	5.6	5	7.5	7.5	6.5
Sartocon 2 (5,850 cm^2)	4.4	6	4.4	4.8	5

Cassettes having small amounts of surface area are subject to the variance in the actual available membrane surface area which can have a significant influence on the observed flux. As area increases these variances become less and less noticeable.

The average standard deviation as a percent of the mean normalized water flux was used to compare the different sized cassettes. As expected, the values increase as the cassette surface area decreases. In the analysis of multiple membrane casting lots from which >10 lots of cassettes were made of various sizes, flux range/standard deviation increased from ± 6% of mean flux for a 0.7m^2 cassette to +/- 8% for 1,000 cm^2 cassettes, and to +/- 22% for 200 cm^2 cassettes (Table 6).

Predicted flux for 10 cassettes is calculated by taking the measured flux from a Slice 200 (200 cm^2) cassette and multiplying it by 10 times the product of the QC based data scale up factors from Table 3 for the Slice 200 to Slice (7.5) and the Slice to Sartocon (4.8). The percentage of error (line 10) is 1- [actual flux for 10 cassettes (line 8) divided by the predicted flux for 10 cassettes (line 9)] (Table 7).

Single Lot versus Mixed Lot Cassette Manufacturing

Single lot versus mixed lot cassette manufacturing strategies can have a significant impact on the statistical analysis of water flux per inter and intra cassette lots. While

TABLE 7 Flux Values for Bovine Serum on a Variety of Cassettes from 200 cm^2 to 6 m^2

	Flux (l/min) vs. TMP				
Cassette/TMP (bar)	1	1.5	2	2.5	3
1 × Slice 200	0.02349	0.0249	0.0253	0.0254	0.0255
1 × Slice	0.1761	0.186	0.19	0.1915	0.1905
1 × Sartocon 2	0.8	0.832	0.856	0.861	0.860
2 × Sartocon 2	1.63	1.74	1.76	1.78	1.78
5 × Sartocon 2	3.68	3.84	3.89	3.91	3.89
10 × Sartocon 2	7.78	8.08	8.25	8.34	8.35
Predicted flux for 10 sartocon cassettes	8.4564	8.964	9.108	9.144	9.18
Error $\% = 1 - \left(\frac{\text{ActualFlux}}{\text{PredictedFlux}}\right)$	8%	10%	9%	9%	9%

mixing membrane casting lots in a cassette lot will result in increased uniformity with regards to both flux and rejection performance, it robs the user from being able to select cassette/membrane lots based on rejection and it blurs regulatory issues regarding traceability. The cGMP concept of lot specificity and component traceability is best adhered to through the use of membrane lot specific cassette lots.

Conclusion

Careful analysis of pressures and scaling factors makes it possible to predict scaled up performance. Scaling predictions should include both average water flux from multiple membrane lots as well as product specific flux.

PROCESS MODES

Crossflow application can be operated in a variety of different modes. These include batch, modified batch and feed and bleed.

Batch

In batch mode the feed solution is added to the Processing vessel and the solution is recirculated through the crossflow system returning to the tank. As the solution permeates the membrane system, the volume concentrates in the feed tank. At the desired level (concentration factor) the system is shut down and the retained contents are is collected or discarded as dictated by the process design (Fig. 37).

Modified Batch/Diafiltration

In a modified batch mode the feed solution (ex. diafiltraiton solution) is added to the Processing vessel and the solution is recircuated through the crossflow system returning to the tank. As the solution permeates the membrane system, new feed solution is added

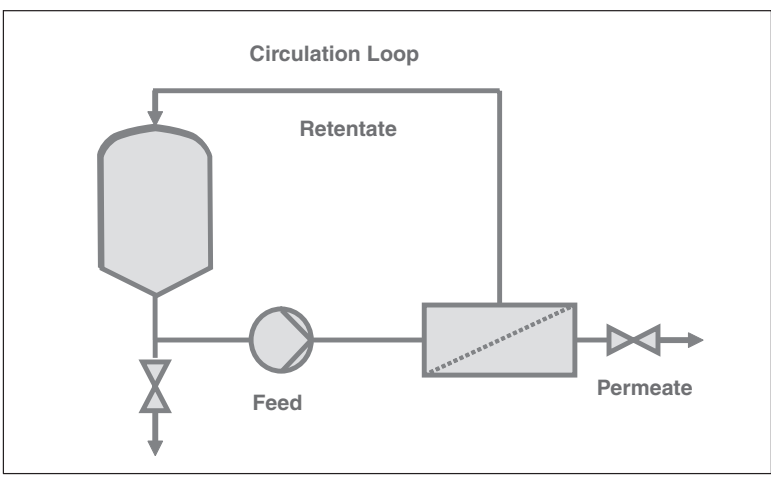


FIGURE 37 Batch mode schematic.

to the feed tank. Feed may be either a constant rate which may or may not be equal to the rate of permeation or in aliquots until the feed/and or the diafiltrate is all transferred to the feed tank. After the transfer is complete the process is completed as in a batch mode and the volume is allowed to concentrates in the feed tank. As before, at the desired level of concentration the system is shut down and the retentate volume is collected or discarded as dictated by the process design (Fig. 38).

Feed and Bleed Batch

Feed and Bleed is used primarily in industrial applications where the volumes are very large and high recirculation rates are required. Generally this mode is not used in cGMP applications. The retentate is recirculated after the feed tank, back to the feed pump (with or without a pressurizing pump in the line. This keeps the recirculation rate down reducing the turn over rate to the feed tank. However, the concentration in the recirculation loop increases. Loop solution must be "bled" back to the bulk feed tank prevent the loop from becoming over concentrated. For example if the loop bleed rate is 10 times the permeate rate the loop volume is only 5% higher than the recirculation tank concentration. This allows for a smaller feed tank, and small 'feed' line to the system from the tank. For large systems with remote tankage this can save quite a lot of large pipe and with a small pressurizing feed pump, a large amount of energy by keeping the loop pressure high (i.e., not dropping it to zero at every pass).

The issue with Feed and Bleed is controlling the loop vs. the return flow rates and cleaning the loop/by-pass lines to ensure proper cleaning levels. For most pharmaceutical and bio-tech applications this process design is not used (Fig. 39).

MEMBRANE CLEANING

It is generally necessary to clean the membranes between filtration batches especially when fouling occurs. Methods employed to clean the membranes must not damage or alter the membrane, and the cleaning agents must be completely rinsed from the membrane prior re-use.

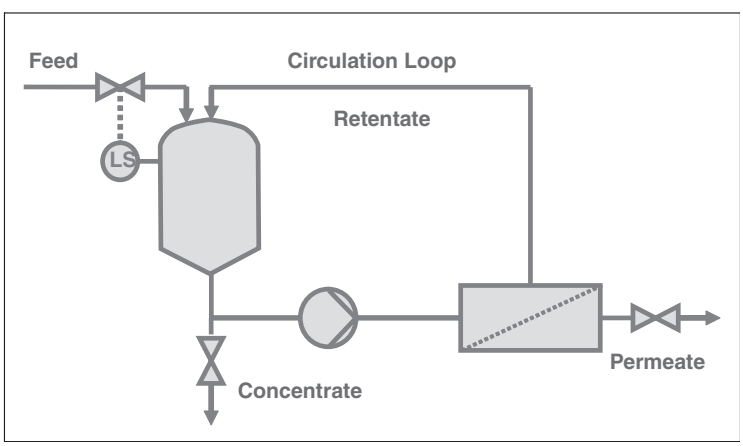


FIGURE 38 Modified batch mode schematic.

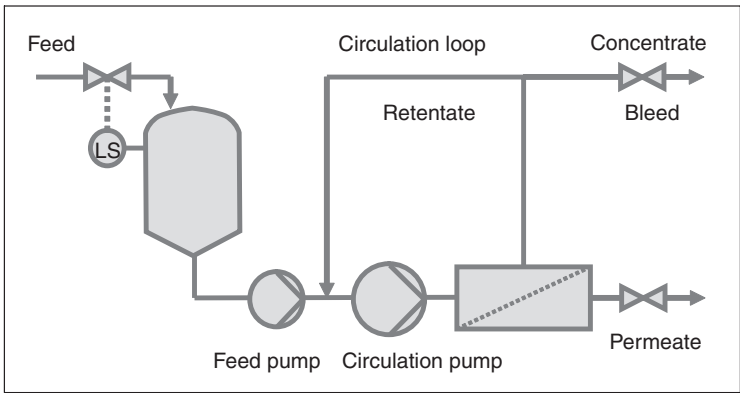


FIGURE 39 Feed and bleed schematic.

The goal of the cleaning procedures is to remove product from the membrane and to assure that there is no cross contamination between production lots and to restore the membrane's flux as close to the original starting flux as possible. The selection of a cleaning protocol must take into account the nature of the product and the agent responsible for soiling the membrane. It is critical to examine the interaction of membrane, the soil and cleaning agent, because it is possible to irreversibly fix fouling proteins to the membrane matrix or to render a membrane unsuitable for the application that it is intended by leaving a residual charge on its surface. Also critical to the process is to be able to test for the presence of any residual cleaning or sanitizing agents because of potentially deleterious affects on subsequent production runs (Kirsch et al., 1993).

Cleaning may be considered from the perspective of time, temperature, and cleaning chemicals. As a rule, the longer a cleaning agent is allowed to work the more effective it is. This is especially true when the cleaning agents contain proteases. The higher the temperature, the more reactive the cleaning agents. The limitation of raising the temperature must be balanced between the reactive nature of the cleaning agent and the stability of the soils (fouling proteins). Too high a temperature can lead to protein denaturation. When proteins become denatured and precipitate on or within the membrane matrix, cleaning becomes considerably more difficult. The proper selection of cleaning chemicals and their proper use will result in the desired effect (Table 8). Just as one must optimize the operating parameters for production, the same can be said for the cleaning process.

The cleaning procedure should begin immediately after the end of the filtration run. Allowing protein-containing solutions to remain in a drained membrane system can lead to protein denaturation and adsorption at the protein-membrane-air interfaces. It is generally advisable to rinse out as much of the product as possible at the end of the run, prior to introducing the cleaning agents. One needs to be cognizant of the solutions used to rinse out the solution because of potential fouling by residual protein or anti-foamants as a result of pH, temperature, or ionic strength. For protein-containing solutions it is generally recommended to rinse the membranes with an isotonic solution.

Historically certain membrane pretreatments have been shown to have profound effects on product yield. Prewashing polysulfone 100,000 MWCO membranes with a buffered bleach solution followed by a 0.1N NaOH rinse gives considerably higher yields in the purification of polio virus than using only the NaOH (Prashad, pers.

TABLE 8 Biological Foulants and Recommended Membrane Cleaning Agents

Foulant	Cleaning agent
1. Anti-Foamant	1. Cold Water and P3 Ultrasil 53 or Calgonite CMR or P3 Ultrasil 11 or P3 Ultrasil 91
	2. Sodium hydroxide
2. Biopolymers	1. Isotonic saline and urea and citrate tetraborate or sodium hydroxide
and	2. P3 Ultrasil 11
3. Proteins	3. P3 Ultrasil 91
4. Blood	1. Citrate solution and isotonic saline
5. Cell debris and	1. Isotonic saline solution
6. Denatured	2. P3 Ultrasil 11 or Calgonite CMR
proteins	3. Sodium hydroxide
	4. P3 Ultrasil 91
7. Lipids	1. P3 Ultrasil 53 or Calgonite CMR
	2. P3 Ultrasil 11
	3. P3 Ultrasil 91
8. Polysaccharides	1. Citrate tetraborate hypochlorite solution
	2. P3 Ultrasil 53 or Calgonite CMR
	3. Sodium hydroxide
	4. Hypochlorite

Commun,). Divizia et al. (1989) improved yields of polio virus by preblocking potential membrane-binding sites using a variety of buffers of varying concentrations (Divizia et al., 1989).

VALIDATION ISSUES

Process-Specific Validation Issues

The compatibility of a membrane system that is reused must look at the effects of reuse on the membranes with regards to any changes in extractables after prolonged exposures to all of the fluids that the process will encounter. These include the storage, rinse, cleaning, sanitizing, and process solutions. Membrane integrity must be monitored by both direct testing of the membrane's integrity as well as by continued achievement of the desired product yield and product quality. Furthermore, the membrane's retention coefficient should also be monitored over the life of the membrane.

Retention coefficients or selectivity measurements are highly subjective. It may be best to specifically determine the membrane's retention coefficient with the products of interest by using them in the presence of normal process fluids. This then provides a practical reference by which to evaluate performance.

3Membrane cleaning and sanitization from a validation stand point must be determined to be effective. It must also be demonstrated that the cleaning agents can be removed prior to reuse. The ability to demonstrate this is important, especially considering the potentially deleterious effects of residuals on product yield and on product purity (Kirsch et al., 1993). Validation of cleaning cycles can be performed through the analysis of permeate and retentate effluents after each stage of the operation. Analysis should include specific assays for compounds that have been introduced into the system. Alternative approaches are to flush the system with WFI

and determine if the effluents continue to meet the WFI specifications. Total organic carbon analysis of effluent rinses offers yet another approach to this problem. Direct physical inspection of membrane systems provides further confirmation that cleaning procedures are adequate. After the membranes have been cleaned it is suggested that a WFI or buffer flux be determined. CWF or buffer flux determinations serve as a benchmark for subsequent cleanings. Generally the flux from a virgin membrane will never be achieved after the first production run followed by the first cleaning. It is not uncommon for the flux to drop 20%, though this reduced value should be retrievable after subsequent use and cleaning cycles.

If the CWF continues to decline after each process run and cleaning cycle, then further or different cleaning techniques need to be investigated. Certain solutions clean well (enzymes, and hypochlorite as examples) but are sometimes difficult to confirm total removal prior to subsequent uses. Continued decline in the CWF indicates the membranes are fouled, or the cassette may be plugging with solids. Fouling may be cleaned, but plugging is almost always irreversible, and care needs to be taken to ensure solids are filtered out before operation or filtered out during operation, or operating parameters changed to prevent precipitation during processing.

Application-Specific Validation Issues

Contaminants

During operation of a concentration process, low level contaminants may suddenly become problematic. Two specific contaminants are of particular interest. First, protein solutions may have low levels of contaminating proteases. Though the rate of proteolysis may be undetectable in the unconcentrated bulk form, after concentration the rate may be increased by the square of the concentration factor (Technical Report No.15, 1992). The effect of proteases on product yield can be devastating to the process. Second, endotoxins may be concentrated from below detectable limits to levels high enough to cause product-lot rejection. Apparent endotoxin content can be also influenced by the nature of the ionic content of the process fluids, which means that they may only appear after a buffer exchange step.

Reuse

Use of "single use" crossflow cassettes should make this separation and process step even more economically and process control interesting to potential users since the time consuming membrane cleaning and validation steps are removed from the process design considerations.

GLOSSARY

Membrane filters are fully characterized by both the manufacturer and the end-user before they are used in the production of a protein pharmaceutical. The methods used in membrane characterization are fully described in this chapter. However, unique terminology is used in the membrane-filtration industry as relates to the characterization and operation of membranes. These terms are defined here to assist those unfamiliar with the membrane-filtration industry.

Adsorption The retention of solutes (typically proteins) to the surfaces of pores in filtration membranes.

Antifoam A chemical added to a solution to prevent or minimize foaming of protein solutions; typically a surfactant.

Back-Pressure Pressure applied to the feed side of the membrane to drive liquid through the membrane.

Batch Process A process where there are no streams flowing into or out of a controlled volume, as opposed to a continuous process. In a batch filtration process, the feed solution is reduced in volume due to permeation of filtrate through the membrane. There is no continuous addition of feed solution to the feed vessel.

Boundary Layer A stagnant layer of liquid against the surface of a membrane that forms due to friction between the membrane surface and the flowing liquid. Higher liquid velocities reduce the thickness of the boundary layer. Boundary layers are observed in crossflow filtration.

Bubble Point A pressure measurement that is directly related to the size of the largest pore in a membrane. A membrane's bubble-point pressure is dependent on the membrane polymer, the membrane wetting solution, and the largest pore in the membrane.

Capsule A dead-end filtration module, typically containing flat-sheet membrane in pleated format. Membrane capsules are ready-to-use units that consist of pleated membrane encased in a molded plastic housing. The entire capsule is a disposable unit.

Cartridge A dead-end filtration module, typically containing flat-sheet membrane in pleated format. Membrane cartridges are placed in stainless-steel housings that come in various designs.

Cassette A crossflow filtration module, typically in a plate-and-frame configuration. Crossflow cassettes are stacked, one on top of another, and placed between endplates.

Centrifugal Filtration A filtration process where the driving force for liquid flow through the membrane is achieved by spinning the membrane device and generating a centrifugal force field, driving liquid outward and through the membrane filter.

Clean-Water Flux A baseline filter flow measurement performed with clean water or buffer, typically measured at 20°C.

Cleaning The process of chemically removing adsorbed, absorbed, or lodged solutes from a membrane.

Concentrate The concentrated feed solution (also known as the retentate solution) after removal of filtered liquid through the membrane.

Concentration Factor The ratio of retained solute concentration after membrane concentration to that before concentration. This factor can be easily determined from the initial feed volume and the final feed volume, assuming that the desired solute is fully rejected by the membrane. $XF = XF/Ci = Vi/Vf$.

Concentration Polarization A situation in crossflow filtration where the rejected solute concentration is much higher at the surface of the membrane than in the bulk feed solution. This occurs when the flowing filtrate solution rapidly drives solute to the membrane faster than the solute can diffuse back into the bulk feed solution.

Crossflow Filtration A membrane filtration process where the feed solution is rapidly circulated parallel to the membrane surface, resulting in minimization of the stagnant boundary layer in the liquid adjacent to the membrane surface.

Dead-End Filtration A membrane filtration process where there is no crossflow of feed and an attempt is made to force all of the feed solution through the membrane.

Diafiltration A crossflow-filtration process where the bulk feed is washed removing solutes and or solvent by continuous or batch-wise addition of buffer to the feed solution, followed by filtration to reduce the total feed volume to its initial volume.

Diffusion Test A test for membrane integrity that involves measuring the rate of gas diffusion through a liquid-wetted membrane.

Dynamic Membrane A layer of rejected solute and/or particulate material that forms at the surface of the membrane. This dynamic membrane can have higher solute rejection than the underlying UF or MF membrane.

Extractables Chemicals used in the manufacture of a membrane or filter that are extracted into the filtered solution during use of the filter.

Feed The solution that is fed to the membrane process.

Filter A porous medium used for removal of specific sized material from a solution; also known as a membrane when the pores are very small.

Filtrate The solution that permeates a filter or membrane; also known as the permeate.

Flowrate The volumetric rate of flow of a solution; expressed in units of volume per time, for example, L/min or gal/day.

Flux The rate of filtrate (or permeate) flow, divided by the membrane area. This parameter is used to characterize membranes. Sometimes "pressure-normalized" flux is reported, which is simply the normal flux divided by the transmembrane pressure driving force.

Fouling The process whereby solute in the feed solution blinds or blocks membrane pores, thereby reducing the filtrate flux.

Integrity Testing The process of determining if a membrane or filter has the reported pore size and solute rejection characteristics. Typically the bubble-point method, diffusion method, or water-pressure integrity test is used to determine the integrity of a membrane.

Log Reduction Value A measure of the efficiency of a sterilizing membrane to retain bacteria, defined as the logarithm (base 10) of the ratio of bacterial count in the feed stream to the bacterial count in the membrane permeate. Typically the Log Reduction Value is reported by membrane manufacturers for retention of Rabdomonas diminuta.

LRV Abbreviation for Log Reduction Value.

Membrane A finely porous medium that is used for removal of specific sized material or solutes from a solution. Also known as a filter when the pores are fairly large–e.g., pores are greater than 0.2 μm in size.

Membrane Area The effective surface area of a membrane that is available for filtration; not the internal pore surface area, but rather the surface of one side of a membrane filter.

MF Abbreviation for microfiltration.

Microfiltration (MF) A membrane-based filtration process where the membrane pore size is in the range of 0.02–1.0 μm. Microfiltration is typically used to separate cells from solutes (e.g. proteins) in the feed solution.

Module A device that combines a large amount of membrane into a compact volume, with easily accessible feed, permeate, and possibly retentate ports.

Molecular-Weight Cutoff (MWCO) A characteristic of membranes or filters, which specifies the average molecular weight of solutes that do not permeate the membrane. This property is dependent on the type and geometry of the solute that is used to characterize a membrane's MWCO.

MWCO	Abbreviation for molecular-weight cutoff.
Nanofiltration (NF)	A membrane-based filtration process where the membrane pore size is 0.0001 μm to 0.002 μm. Nanofiltration is typically used to concentrate fairly small molecules from water.
NF	Abbreviation for nanofiltration.
Optimum TMP	The transmembrane pressure (TMP) that maximizes the overall throughput of a membrane-filtration process. The optimum TMP should be independently determined for each unique filtration.
Permeability	The liquid flux through a membrane which has been normalized to the pressure driving force or TMP.
Permeate	The solution that permeates a filter or membrane; also known as the filtrate.
Plate-and-Frame	A membrane-module geometry where membranes are sandwiched together one on top of another.
Polyethersulfone	A polymer used for formation of many MF and UF membranes.
Pore Size	A characteristic of a membrane that specifies the size and retention-characteristics of a membrane's pores. Typically the absolute pore size is determined from integrity tests, such as bubble point tests for MF membranes and polymer-exclusion tests for UF membranes.
Pore-Size Distribution	The range of pore sizes in a membrane, which is centered around the membrane's average pore size.
Porosity	The percentage of a membrane's volume that is occupied by air in the membrane's pores.
Recovery	The mass of desired solute in the final product solution (either permeate or retentate, depending on the process), divided by the mass of desired solute in the initial feed solution, expressed as a percentage. Recovery is synonymous to yield.
Rejection	The ability of a membrane to exclude solutes or particulate matter from passing a membrane.
Retentate	The liquid stream, concentrated in solute, that exits a crossflow membrane device. By mass balance, the flowrate of the retentate stream equals the feed flowrate minus the filtrate flowrate.
Retention Efficiency	The ability of a membrane to retain a specific solute, expressed as a percentage. Often synonymous with rejection.
Reverse Osmosis (RO)	A membrane-based filtration process where the membrane rejects salt from solution. Pore size is not entirely meaningful for reverse-osmosis membranes, as pores are often not observable by microscopic methods. Reverse osmosis is used to desalinate water.
Shear Force	A stress on solutions that results from high velocity gradients (or differences) in solutions. A solution's viscosity is a measure of that solution's ability to resist deformation when exposed to shear forces.
Stirred-Cell Filtration	A surrogate for crossflow filtration where shear is achieved by rapidly stirring the solution immediately adjacent to the membrane. Typically the stirring is accomplished by mechanical means, such as through the use of a stir bar or impeller.
Tangential-Flow Filtration	Another term for crossflow filtration.
Throughput	The volume of solution that can be filtered through a specified area of membrane before filtrate flow is reduced to near zero.
TMP	Abbreviation for transmembrane pressure.
Transmembrane Pressure (TMP)	The pressure difference equal to the average feed-stream pressure minus the average filtrate-stream pressure. TMP = $(P_{feed} - P_{retentate})/2 - P_{filtrate}$.

UF	Abbreviation for ultrafiltration.
Ultrafiltration (UF)	A membrane-based filtration process where the membrane's pore size is in the range of 1000 to 1,000,000 Da MWCO. Ultrafiltration is typically used to separate and concentrate proteins or other high molecular weight solutes from a liquid solution.
Velocity	The linear rate of solution flow, equal to the solution flowrate divided by the cross-sectional area of the flow conduit. Crossflow velocity is a key parameter in determining the effectiveness of a crossflow-filtration operation.
Viscosity	A solution property that is a measure of the fluid's resistance to deformation when acted upon by shear forces. Higher protein concentrations generally lead to solutions with higher viscosity.
Yield	The mass of desired solute in the final product solution (either permeate or retentate, depending on the process), divided by the mass of desired solute in the initial feed solution, expressed as a percentage. Yield is synonymous to recovery.

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20

Ensuring Safety of Biopharmaceuticals: Virus and Prion Safety Considerations

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Viruses were so named after the Latin for poison. Among the various descriptives used to characterize viruses, "filterable" was a key attribute. Over the past three decades, there have been phenomenal advances in membrane filtration technologies that have allowed development of membranes that retain viruses by a mostly size-exclusion based mechanism. Size-based (predominantly) filtration-removal of viruses is similar to removal of other viable or non viable particulates (and several chapters in this book have been dedicated to its discussion). The challenge lies in the fact that many therapeutic proteins, either native or recombinant, are at the size limit of small viruses; consequently, membrane filtration for virus removal must remove viruses of concern without significantly compromising product yield.

The biotechnology industry has experienced considerable technology maturation in the last several decades. Expert reports inform us that the biotechnology industry is currently alive and well. Big pharmaceutical companies forecast about 60% of revenue growth to come from biologic products (Datamonitor, www.datamonitor.com). The American biotechnology industry has surpassed pharmaceutical companies for the third straight year as the primary source of new medicines; biotech revenue jumped nearly 16% to a record of $50.7 billion in 2005 (Ernst and Young LLP, 2006, www.ey.com). According to a report released by the Pharmaceutical Research and Manufacturers of America (PhRMA; www.phrma.org), in August 2006, 418 medicines and vaccines—developed through biotechnology—are being tested to treat more than 100 diseases. Understandably, biotechnology is one of the most research-intensive industries in the world. The U.S. biotechnology industry spent $17.9 billion on research and development in 2003 [Biotechnology Industry Organization (BIO), 2006, www.bio.org].

VIRUS SAFETY OF BIOPHARMACEUTICALS: GENERAL CONSIDERATIONS

First generation biopharmaceuticals were introduced in the early to mid twentieth century. These therapeutics were sourced from mammalian fluids (e.g., plasma-derived coagulation factors or immunoglobulins, hormones sourced from human urine, bovine-sourced heparin) and tissues [e.g., human growth hormone (hGH) sourced from the pituitary gland of human cadavers, placenta-derived bovine products such as albumin and collagen]. As technology advanced, "second generation" therapies were introduced; these

were directed at selective alteration of the naturally occurring protein to facilitate increased production of the target protein in order to make the manufacturing process commercially feasible, for example, monoclonal antibodies and recombinant proteins. "Third generation" therapies such as gene manipulation are targeted to directly access the patient. While currently development of third generation therapeutics has stalled due to ethical and legal concerns, they hold significant potential in the years to come.

The incidence of virus transmission via plasma-derived (first generation) biologicals has decreased considerably in the last two decades; however, unfortunately, iatrogenic accidents continue to occur. In spite of donor screening and incorporation of processing steps with potential to effect virus clearance, virus has been detected in plasma pools, and plasma-derived coagulation factors (Factors VIII and IX) have been responsible for transmission of HIV, hepatitis viruses, and parvovirus (Blume et al., 2002; Nubling et al., 1995; Santagostino et al., 1997; Scheiblauer et al., 1996; Wu et al., 2005; Chudy et al., 1999; Schneider et al., 2004; Willkommen et al., 1999). While adventitious viral contamination of cell culture harvests has been reported (Garnick, 1996; Rabenau et al., 1993), fortunately, there have been no cases of viral transmission by these recombinant proteins. Table 1 is a listing of virus abbreviations used in this chapter.

Donor selection, testing of donations and of plasma pools for specific relevant viruses (HBV, HCV, HIV, and HTLV) are regulatory mandates designed to reduce the infectious virus burden in the plasma pool used in the manufacturing process. However, the risk of virus transmission via biologicals cannot be completely mitigated by this single approach. It is impossible to screen for "all" infectious viruses, and relevance of pathogens to plasma-derived products is an evolving concept. For example, in the last century, blood-borne pathogens with relevance to transfusion and plasma-derived products were HBV, HCV, and HIV types 1 and 2; at that time human parvovirus B19 and HAV were classified as "occasionally relevant". Today, non-enveloped viruses such as B19 and HAV are of greater concern. New and emerging viruses will also continue to challenge the safety of our blood supply; recent examples include West Nile Virus (WNV) and SARS coronavirus (Kempf et al., 2006). Another consideration is that plasma-derived products are exported across continents; this makes the potential for transmission of a viral agent, non-indigenous to the geographically distant location, a global concern.

TABLE 1 List of Virus Abbreviations Used in this Chapter

BPyV	Bovine Polyoma virus
BVDV	Bovine viral diarrhea virus
CPV	Canine Parvovirus
EMCV	Encephalomyocarditis virus
HAV	Hepatitis A virus
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HTLV	Human T-cell lymphotropic virus
IBR virus	Infectious Bovine Rhinotracheitis virus
PI-3 virus	Parainfluenza-3 virus
SARS Coronavirus	Severe acute respiratory syndrome Coronavirus
WNV	West Nile virus

The wisdom of the current holistic approach to virological safety was put to the test when WNV transmission was reported in 2002 via blood transfusions and organ transplants. Crisis management was immediately incorporated in the blood transfusion industry. WNV was less of a crisis to the plasma industry primarily because plasma products undergo several purification steps that have the potential to effect viral clearance. Additionally, many manufacturers include a virus (Bovine Viral Diarrhea Virus) belonging to the same virus family as WNV in the test virus panel when conducting viral clearance evaluation (validation) studies.

Zero risk is a myth; currently, it is impossible to establish "absolute" virological safety and safety can only be addressed from a risk mitigation and management standpoint (Aranha, 2001a). Several factors account for this reality. Viral assays lack the sensitivity to detect titers, which, although low, may be of medical concern. Also, currently, infectivity assays are not capable of determining total virus titer. Because of the diversity of viruses a specific assay must be performed for each virus. Direct testing for the absence of viral contamination from a finished product is not considered sensitive enough for detection of low levels of virus. Multiple approaches are therefore used to minimize and manage virus contamination risks. Table 2 summarizes the unique virus-contaminated-related issues.

This chapter discusses issues to address in designing and incorporating adequate viral clearance strategies during the production of biologicals and biopharmaceuticals. While the focus is on safety assurance of continuous cell line (CCL)-derived products, the safety and procedural considerations as applied to plasma-derived products are also addressed. A brief section on issues related to safety of biologicals from a prion standpoint is also included.

TABLE 2 Issues Unique to Viral Contamination of Biopharmaceuticals

Virus-related	Infectious potential of even low levels of virus (e.g., HIV)
	Efficient amplification of low levels of virus in cell substrates or product recipients
	Unknown amphitropism of unknown viral variants
Virus detection method-related	Limited sensitivity of virus detection methods
	Sampling-associated logistic limitations, ie, inability to assay large volumes
	Most virus assays are highly specific
	Great diversity of viruses (and, consequently, the necessity of performing specific assays for each virus); there is no "general purpose" growth system that can be used to assay a variety of viruses (as is the case with detection of bacterial contaminants in pharmaceutical production)
Source material-related considerations	Blood donations during the window period, ie, the donor is infectious but routinely mandated testing fails to detect infectious virus
	In the case of plasma-derived biologicals, presence of neutralizing antibody in the plasma pool can mask detection of low levels of infectious virus
	Some source materials (e.g., cell lines used for biopharmaceutical manufacture) may harbor non-infectious retroviral particles; safety of the recombinant proteins from these substrates must be evaluated using a risk-based approach

CONTROL OF PRODUCTION PROCESSES FOR VIRAL SAFETY

Both regulatory groups and the pharmaceutical industry acknowledge that the quality of a finished dosage form is directly impacted by the constituents used in production. Appropriate sourcing gains even more importance in the area of clearance of viruses and unconventional agents (e.g., prions) where it is currently impossible to test for and claim complete absence of infectious biological agents in any given product.

Ideally, all raw materials used in manufacturing processes must be carefully controlled; however, by its very nature the source pool for plasma-derived products is highly variable and constantly changing. Blood donors enter/leave the pool at will; their health status is also not static. While blood is screened for specific pathogenic viruses (HIV, HBV, HCV, and HTLV), low levels of virus may escape detection by currently used methods due to a variety of reasons: donations may be during the "window period" when the person is infectious but the screening test does not detect the virus; presence of high levels of neutralizing antibodies to a particular virus in the plasma pool (which is not controllable) is another factor that has contributed to transmission of viruses such as HAV and parvovirus B19 via contaminated coagulation factor concentrates.

In the case of biotechnology-derived products, hybridoma and recombinant technology are now mainstream. They allow the production of high levels of the target protein to make the process commercially feasible. Some biotech products have their origin in research projects. In vivo production of therapeutics (e.g., a monoclonal antibody derived from ascites produced in mouse peritoneal cavities) was a routine practice several decades ago and is still sometimes used in research laboratories during the early stages of the development lifecycle. In general, the uncharacterized nature of ascites fluid is currently unacceptable and in order to progress to commercial production an established cell bank that is properly qualified and safety tested is required.

For recombinant products, a two-tiered system in terms of establishment of a master cell bank (MCB) and working cell bank (WCB) following cGMP is the first step in ensuring the quality of a biopharmaceutical product (ICH Q5D, 1997). As cell banks are extensively characterized, any viral contaminant associated with them will not be cytolytic; however, chronic or latent viruses could potentially be present. Endogenous retroviruses and retrovirus-like particles are associated with some CCLs; they are non-infectious but pose a theoretical safety concern due to their oncogenic potential. The widespread use of murine cell lines in the manufacture of monoclonal antibodies is another potential source of introduction of rodent zoonotic agents. Chinese Hamster Ovary (CHO) cells, a cell line frequently used in monoclonal antibody production, may harbor contaminants such as Hantavirus. Monoclonal antibodies produced in human/humanized (human/mouse) cell lines are preferred from an immunological standpoint; however, due to the absence of a species barrier they raise unique viral safety considerations. Humanized cell lines are derived from human B lymphocytes, which can harbor several viruses including Retroviruses, Hepatitis viruses, Human Herpes viruses, Cytomegalovirus and Human Papilloma virus. While currently not done, use of specific viral agents, for example, Epstein-Barr virus or Sendai virus, for cell line establishment or cell transformation could also contribute to the viral load.

Viral contaminants may also be introduced adventitiously via the additives used/manipulations undertaken in production (Castle and Robertson, 1998). The manufacturing process for a recombinant protein, for example, may include use of constituents such as albumin, transferrin, Tween-80, lipoproteins, and gelatin that are bovine sourced. Cell lines are often cultivated in serum-supplemented media (5–10% serum) or reduced serum media

(2–4%). Bovine viral diarrhea virus (BVDV) has been identified as the most common contaminant of bovine serum. Other possible contaminants of bovine serum include Reovirus, Infectious Bovine Rhinotracheitis virus, Parainfluenza-3 virus (PI-3) Bovine Leukemia Virus and Bovine Polyoma Virus. Porcine parvovirus is reportedly a common contaminant in preparations of porcine trypsin used for the preparation of cell cultures. While serum-free media is the growth medium of choice, it must be noted that serum-free medium and mammalian supplement-free medium are not synonymous (Brorson et al., 2005); for example, a chemically defined medium may be supplemented with recombinant growth factors produced in a serum-supplemented system. A preparation designated "protein-free" may not contain protein but filtered protein hydrolysates.

The manufacturing history of the raw material has come under increased scrutiny in the last decade. For example, yeast used for the production of yeast extract—a common additive in production processes—may be grown in a medium containing animal-derived peptones. A claim that the amino acid derived in a recombinant yeast system that contains yeast/meat extract as an additive has not been exposed to human/animal-derived components is not valid. Similarly, a chemically derivatized animal-sourced amino acid salt is not a synthetic amino acid. Table 3 presents a general classification of raw materials used for manufacturing biotechnology-derived products.

Purification processes also contribute to the viral load. For example, affinity chromatography using monoclonal antibodies as ligands increases the potential for adventitious virus introduction into the product. Other ancillary sources of viral burden include breach of GMP and consequent virus introduction from manufacturing environments or personnel; these viral contaminants would not be removed by conventional "sterilizing-grade" filters, which are intended for removal of bacterial and microbial contaminants other than viruses. The potential for viral contamination for each of the manufacturing unit operations must, therefore, be evaluated and its impact on the viral load assessed.

VIRUS DETECTION METHODS

Virus assays should have adequate sensitivity and reproducibility and should be performed with sufficient replicates and controls to ensure adequate statistical accuracy of the test results.

TABLE 3 Classification of Raw Materials Used In Manufacture Of Biologicals And Biopharmaceuticals

Source	Examples
Directly sourced from animal	Bovine serum albumin (BSA), transferrin, porcine trypsin used in production; human serum albumin used as an excipient during production of recombinant proteins such as Factor VIII
Indirectly animal-derived—fermentation product manufactured in a system supplemented with animal-derived components	Recombinant proteins produced in a bacterial/yeast system supplemented with additives like beef/meat extract
Secondarily animal-derived: material is manufactured in a process supplemented with materials which are indirectly animal derived	Yeast extract used in a fermentation process, e.g., amino acids, where the yeast was grown in a meat extract supplemented medium

There are several available virus detection methods: infectivity assays (in vitro and in vivo), molecular probes, biochemical assays, morphological assays, and antibody production tests (in animals). The particular viral detection method used will depend on the objective of the test, what is being tested and other issues. For example, the effectiveness of a virus clearance unit operation is commonly assessed using infectivity tests, though validated polymerase chain reaction (PCR)-based assays are being increasingly used. To estimate the non-infectious retroviral burden, assays of choice include morphological assays (electron microscopy) or biochemical assays.

Infectivity assays are the gold standard and essentially involve inoculation of susceptible cell lines with the specific virus, followed by monitoring and observation of cytopathic effects, for example, formation of plaques, focus forming units or induction of abnormal cellular morphology, as a consequence of the infection. The two types of in vitro infectivity assays commonly used to estimate viral titer are the plaque (or focus) forming assay and the 50% tissue culture infectious dose ($TCID_{50}$) assays. The plaque-forming assay offers extreme sensitivity and is especially useful when the virus is present at extremely low titers. $TCID_{50}$ is defined as that dilution of virus required to infect 50% of a given batch of inoculated cell cultures. Both the plaque assay and the $TCID_{50}$ assay have been extensively validated for use in process clearance evaluation (validation) studies. While infectivity-based assays are favored due to their extreme sensitivity and specificity, the requirement for a different assay system for each virus (due to the cell culture-specific infectivity) makes biological assays cumbersome.

Molecular probes such as hybridization assays or PCR assays are being increasingly used because of their specificity and the rapidity of the results. These methods, in general, detect the presence of nucleic acid (DNA/RNA) but cannot differentiate between infectious or non-infectious particles. Additionally, the method is applicable only when the genomic sequence of the virus is known, as in the case of retroviral genomes. PCR is especially relevant either if the viral agent cannot be grown in vitro, for example, type A retroviral particles, or, for viruses such as Hepatitis B and C where there are severe limitations to culturing them in vitro. Additionally, for example, if a unit process operation, such as chromatography, is able to effect both virus removal (resin-associated) and inactivation (low pH inactivation by eluting buffer), PCR allows for differentiation of the contribution of each of the mechanisms. With the availability of fluorogenic 5'-nuclease-based Q-PCR, it is possible to undertake a multi-virus spike in a single preparation and measure the clearance achieved simultaneously in separate assays (Valera et al., 2003). A note of caution: while methods such as PCR have provided enhanced assay sensitivity, a negative PCR result does not prove unequivocally the absence of virions (infectious or not), due to the effect of sample size and it's impact on assay sensitivity.

Morphological assays such as electron microscopy, while of limited value to assay viral load in fluids, are especially relevant for estimation of viral load in cell lines containing non-infectious particles, such as the Type A retroviral particles, which are present in several rodent cell lines used in biotherapeutics production.

Biochemical assays such as reverse transcriptase assays, radiolabel incorporation into nucleic acids, radioimmunoassays, immunofluorescence and Western blots are also used for virus detection. However, these tests are semi-quantitative; also they detect enzymes with optimal activity under the test conditions and their interpretation may be difficult due to the presence of cellular enzymes or other background material.

Among other tests required for characterization of the murine hybridoma or other production cell lines at the MCB establishment stage, the antibody production test is used.

Mouse antibody production tests, hamster antibody production tests, and rat antibody production tests allow detection of viruses which may be associated with the cell line and have the potential for infecting humans and other primates. Alternatives to animal testing are being investigated.

It is important to recognize that while positive results are meaningful, negative results are ambiguous. This is because it is not possible to determine whether the negative result reflects inadequate sensitivity of the test for the specific virus, selection of a test system (host) with too narrow a specificity, poor assay precision, limited sample size, or basically, just absence of virus. This is highlighted in cases where limited sensitivity of the screening methods, combined with masking of presence of infectious virus by neutralizing antibody in the plasma sample pool, have resulted in iatrogenic viral transmission via contaminated plasma products (Blumel et al., 2002; Chudy et al., 1999).

REGULATORY CONSIDERATIONS: A RISK BASED APPROACH

Clinical acceptability of biologicals and biopharmaceuticals, must, of necessity, be guided by risk-benefit analysis (Aranha 2004). Risk assessment involves process analysis to identify sources of risk and their consequence. In view of the unique considerations associated with viral contaminants (i.e., actual versus theoretical presence) and the limitations in the assay methodologies (inability to establish absolute absence of viral presence), regulatory agencies emphasize a holistic approach directed at risk minimization, which, when combined with process monitoring, constitutes an appropriate risk management program.

All guidelines and regulatory documents distinguish between well-characterized biologicals (where viral contamination is often a theoretical concern) and traditional products such as blood derivatives where there is a significant potential for viral presence (e.g., parvovirus B19, Hepatitis viruses, HIV). Thus, for example, low levels of infectious virus in plasma products are prohibited and any virus-contaminated source material would be immediately quarantined. However, in the biotechnology industry, cell lines such as CHO cells containing endogenous retrovirus, at levels of 10^6–10^9 particles/ml (as visualized by electron microscopy), are deemed acceptable as the particles are non-infectious and pose primarily a theoretical safety concern.

In general, the major factors influencing the viral safety of biologicals are the following: (*i*) the species of origin of the starting material, that is, non-human viruses are less likely to initiate infection in humans due to species specificity of these viruses; the species barrier, however, is not absolute; (*ii*) the degree of source variability of starting material, (e.g., human plasma-derived products which are manufactured from pooled donations pose a higher risk compared with products derived from a well-characterized cell bank) and the possibility of testing the source material for the presence of viral contaminants (feasible for blood donation but not feasible for animal-derived products), (*iii*) the purification and processing steps and their capacity for viral burden reduction, and (*iv*) the existence of specific steps for viral clearance included in the process.

The current risk minimization strategy to guard against inadvertent virus exposure of patients treated with a biological is a combination of three efforts: (*a*) prevention of access of virus by screening of starting materials (cell banks, tissues, or biological fluids) and raw materials/supplements used in production processes (culture media, serum supplements, transferrin, etc); (*b*) incorporation of robust virus clearance steps and (*c*) monitoring production using a relevant screening assay. Table 4 presents currently used

TABLE 4 Risk Minimization Strategies for Virus Contamination Control

Barriers to entry, i.e., appropriate sourcing	Characterization of master cell bank and working cell bank Selection and screening of donors
Incorporation of "robust" virus clearance steps	Serendipitous/deliberate virus removal/inactivation steps, e.g., filtration, chemical inactivation, etc.
Testing during production to ensure absence of contaminating virus	Virus detection assays such as infectivity assays, PCR, etc during manufacture

risk minimization strategies. Engineering and procedural control over facilities, equipment and operations, as required by cGMP, are an important component of the safety paradigm.

How Much Viral Clearance is "Enough"?

While the necessity for risk assessment and incorporation of not merely adequate but excess virus clearance capacity is acknowledged, the amount of excess capacity required has not been clearly defined. Industry wisdom suggests that manufacturing processes must be validated to remove or inactivate ≥4–6 orders of magnitude more virus than is estimated to be present in the starting material. Extrapolating from the "Sterility Assurance Level," applied to bacterial sterility considerations, of not more than one viable microorganism (10^{-6}) in 1×10^6 volume of final product, a 6 $\log_{10}$ safety factor is routinely considered adequate. In the case of plasma-derived biologicals, there will not be any detectable baseline viral load (as source materials with detectable virus contamination would be immediately quarantined); for products derived from CCLs known to harbor (non-infectious) endogenous retroviruses, it is necessary to determine the theoretical viral burden per dose equivalent of the biological product and incorporate an appropriate safety margin (6 $\log_{10}$).

A key factor affecting the overall process clearance factor required for a product is the amount required to produce a single dose of product. The required level of clearance is assessed in relation to the perceived hazard to the target population and is guided by risk benefit analysis. For example, CHO cell lines containing endogenous retroviruses are deemed acceptable if the manufacturing process can be demonstrated to provide adequate retrovirus clearance. The clearance goal is usually chosen based on the product use and the risk to the patient population. The extent of product testing necessary will depend on the source and nature of the product, the stage of product development, and the clinical indication.

Risk calculations to determine retroviral load per dose are shown in Table 5. This example assumes a one-time dose of 1200 mg to the patient. To achieve a conservative goal of a probability of a viral contamination event of 1 particle per million doses of product, and assuming a retroviral load of 1.62×10^7 particles/ml in the start material, the purification process for this product would have to demonstrate a minimum log clearance of 16.85 logs to achieve the stated goal of 1 viral particle /10^6 doses (Aranha and Forbes, 2001).

Biopharmaceutical safety is the result of multiple orthogonal barriers operating in concert. While each approach, individually, may have limitations, their use in an integrated manner provides overlapping and complementary levels of protection from

TABLE 5 Risk Calculation to Determine the Viral Load Per Dose

Retrovirus-like particles/ml	1.62×10^7 particles/mL
Antibody titer	0.274 mg/mL
Weight of average person	80 kg
Dose per mass	15 mg/kg
One dose	1200 mg
Viral clearance factor	Unknown

The total amount of retrovirus-like particles in one dose = [$(1.62 \times 10^7$particles/mL)(1200 mg/dose 0.274 mg/mL)] ÷ 10^{-6}particles/dose = 7.09×10^{16} or 16.85 logs minimum clearance required to achieve a clearance of 1 particle per million doses

Challenge virus: Xenotropic Murine Leukemia virus (X-MuLV)

Source: Aranha and Forbes, 2001.

putative viruses to recipients of recombinant and monoclonal products. To quote from ICH-Q5AR1 (ICH Q5AR1, 1997): "Confidence that infectious virus is absent from the final product will in many instances not be derived solely from direct testing for their presence but also from a demonstration that the purification regimen is capable of removing and/or inactivation of the viruses." Multiple orthogonal approaches for virus removal and inactivation are more effective than single steps.

VIRUS CLEARANCE METHODS

An ideal clearance method should be robust and have a broad spectrum of clearance (either through inactivation or removal) of viruses (both enveloped and non-enveloped), concomitant with high product recovery. The method should be minimally invasive and non-contaminating, ie, should not involve addition of stabilizers or other additives which must be removed post treatment, and should not alter the biological integrity or reactivity of the product. The mode of action should be well characterized and the method should be scaleable and amenable to process validation (clearance evaluation).

Suitability and choice of a particular clearance method will be guided by the following considerations: the characteristics of the product, that is, the size of the protein, its conformation, it's lability to heat or other inactivation methods; the characteristics of the potential viral contaminants, that is, viral size, lability, presence/absence of a particular macromolecule such as a lipid envelope; and process evaluation considerations, that is, logistics at process scale, scale-up, and scale-down considerations for process evaluation (validation), etc.

Viral clearance may be achieved as a consequence of routine processing and purification operations or strategies specifically aimed at viral clearance may be incorporated into the manufacturing process. Serendipitous (or fortituous) virus clearance methods are operations that are part of the product purification process that offer the added bonus of viral clearance; these methods are not optimized for viral clearance but co-incidentally provide viral clearance. Methods commonly used in the purification of biopharmaceutical products (clarification, centrifugation, extraction, precipitation and filtration; and affinity, ion-exchange, gel-filtration, hydrophobic interaction, and mixed-mode exchange chromatography) may physically separate virus particles from the product (virus removal) based on size, charge, density, binding affinities, and other differences between the virus and the product. Similarly, viral inactivation may occur as a consequence of pH effects during processing, use of low pH buffers for elution of

proteins from chromatography columns, and, inactivation by reagents used in the purification process. In addition to the serendipitous virus clearance afforded by downstream purification options, methods deliberately introduced for virus clearance, eg, heat inactivation, solvent-detergent inactivation, virus filtration, are also commonly incorporated during manufacturing.

Clearance efficiency is evaluated in terms of the $\log_{10}$ reduction value (LRV) which is the ratio of the viral concentration per unit volume in the pre-treatment suspension to the concentration per unit volume in the post-treatment suspension. Other synonyms for LRV are $\log_{10}$ titer reduction (LTR) and $\log_{10}$ reduction factor.

Virus Removal and Inactivation Methods

Depending on their mode of clearance, virus clearance methodologies are classified as virus removal strategies which aim at (mechanical) reduction of viral numbers, or virus inactivation methods where the objective is irreversible loss of viral infectivity. Virus inactivation steps must not compromise a product's stability, potency, biochemistry, or biological activity. The inactivation strategy used will be dictated by the following considerations: lability of the virus, the stability of the biological preparation, and the effect on other components in the preparation.

Inactivation methods are very effective in decreasing the viral burden; however, there are limitations. Heat treatment can denature certain proteins. Stabilizers, (sometimes added during inactivation by heat or solvent-detergent, to ensure that the biological activity of the active moiety is not compromised), may be protective, not just to the target protein but to the virus as well. In addition to protein denaturation that may occur, the viral inactivation method has the potential to alter the functionality or antigenicity of either the active ingredient or other proteins in the product (Suontaka et al., 2003; Peerlinck et al., 1993).

One of the important considerations to be addressed in virus inactivation experiments involves evaluation of the kinetics of virus inactivation. This is important since virus inactivation is rarely linear and a persistent fraction can exist which is more resistant to inactivation than the majority of the virus population. Certain process parameters may critically impact viral clearance. Savage et al. (1998) reported a minimum threshold moisture level requirement for efficient virus inactivation to occur during dry heat treatment of freeze-dried coagulation factor concentrates; similarly, presence of cations have been demonstrated to contribute to the thermostability of viruses (Melnick, 1991).

Extrapolation of the potential for virus clearance of any given method to other viruses (e.g., within the same virus family or based on a physical characteristic such as size) must be done with extreme caution and will depend on the virus clearance method and the mechanism of virus clearance. For example, it is reasonable to infer that a virus removal filter specifically designed to remove small viruses ($\geq$20–25 nm) will be effective in removal of large viruses as well (viruses such as the hepatitis viruses, HIV, PRV). However, in the case of virus removal by partitioning or chromatography, it is not possible to extrapolate virus behavior even within the same virus family as the physicochemical properties of the virus impact removal. In inactivation studies, different susceptibilities to low pH treatment have been demonstrated in Murine Minute Virus and parvovirus B19 (Boschetti et al., 2004). In terms of heat inactivation, in general, B19 is more sensitive to heat compared with other parvoviruses (CPV, PPV) (Prikhodko, 2005; Blumel et al., 2002; Yunoki et al., 2003, 2005). However, B19 heat sensitivity was shown

to be dependent on other factors—heating conditions, that is, dry heat, heating in liquid, pasteurization (Yunoki et al., 2003).

Combinations of methods, as, for example, UV irradiation/-propiolactone (Lawrence, 2000), and use of psoralens in combination with long-wavelength ultraviolet light (UVA, UVC) (Knutson et al., 2000) have also been evaluated. Additional technologies are constantly being developed, as, for example, inactivation with compounds such as biosurfactants (Vollenbroich et al., 1997) and Inactines™ (V.I. Technologies, Watertown, MA/Pall Corporation, NY) (Suontaka et al., 2003) and application of pressure cycling technology (Bradley et al., 2000) for virus inactivation.

Filtration For Viral Clearance

The first filter specifically designed for viral particle removal from biopharmaceutical products was the Planova® filter introduced in 1989 (Manabe et al., 1989). Currently, several virus filters capable of removing "large" and "small" viruses are available. Until recently, the process of using filters specifically designed for virus removal was referred to as nanofiltration; however, in the interest of accuracy, the term "virus filters" has replaced the incorrect "nanofilters" used earlier to refer to these filters.

As with sterile filtration, virus (and non-viable contaminant) retention by membranes designed specifically for virus removal is affected by membrane attributes, ie, morphology of the membrane (pore shape and uniformity), pore size and membrane thickness as well as by ancillary factors including the kinds of interactive forces between contaminants and the filter membrane (e.g., inertial impaction and diffusional interception due to Brownian motion). In general, particulate removal by membrane filters occurs via either size exclusion or adsorptive retention. Size exclusion-based removal (the mechanism of choice) occurs due to geometric or spatial restraint, and, theoretically, is not directly influenced by either process-related (e.g., differential pressure, temperature, flux) or product-related (e.g., kind of protein and its concentration, viscosity, ionic strength, pH, surface tension) considerations. With adsorptive retention, other factors may influence removal, that is, filter membrane composition and electrokinetic/hydrophobic interactions with the membrane, and filtration conditions (flow rate, pressure, temperature). These mechanisms, that is, size exclusion and adsorptive retention, are not mutually exclusive; they operate concomitant with each other but the relative importance and role of each may vary, depending on the filter type. There is considerable evidence supporting the reliable and robust performance of virus filters. To quote the technical report on virus filtration released by the *PDA Journal of Pharmaceutical Science and Technology* (TR-41) (PDA, 2005): "Virus filtration is performed as part of a manufacturer's overarching virus safety strategy."

The two major membrane filtration systems are single pass, "dead end" or direct flow filtration (DFF) and cross flow or tangential flow filtration (TFF). The flow path in DFF is perpendicular to the filter surface and viable and non-viable particulates being filtered out are deposited both on and within the depth of the membrane. It is, therefore, not possible to quantitatively recover virus trapped within the depth structure of the membrane, and accurate mass balance for DF filtration cannot be established. The advantages of DFF include ease and speed of use, low shear and high levels of product recovery; however, due to their depth style construction, DF filters require relatively clean process fluids as particulate penetration and clogging of the pores can occur. When using DF filters, one approach to optimize process economics is to use pre-filters to extend the life of the virus filter. In the TFF mode, the liquid flow path on the upstream

side of the filter is tangential to or across the filter surface; a portion of the liquid passes through the membrane surface (permeate), while the rest is returned to the central reservoir as the retentate. In TFF, the volume of fluid in the retentate continually decreases as more of the initial volume is collected as permeate. Particulates (viable and non-viable) are concentrated in the retentate. TFF membranes can handle higher particulate loads. While TF filters are theoretically reuseable after cleaning and sanitization, re-use is not the norm with virus-removal filters because of the complexity of the validation issues involved.

Filtration for virus removal has several advantages. It does not compromise the biological integrity of the product thereby inducing adverse biological and immunological reactions; both in vitro and in vivo studies (Burnouf-Radosevich et al., 1994; Mannucci and Tradati, 1995) confirm that, in general, filtration does not induce alterations of plasma proteins (increased immunogenicity, formation of neoantigens). The non-invasiveness and non-destructiveness of filtration is attributed to the relatively mild physiological conditions (pressure, pH, osmolarity and temperature) under which the operation is carried out. When viral particle removal is based on size exclusion, filtration constitutes a "robust" mechanism for virus removal, as it is not unduly influenced by minor alterations in process parameters.

When evaluating a filter for a virus removal application, the clearance mechanism must be ascertained, that is, whether removal is primarily based on size exclusion or whether other dominant factors come into play, that is, formation of a gel layer on the membrane surface (which effectively results in a smaller pore size, and, consequently, enhances viral retention) or whether removal is effected by a charge-based mechanism (i.e., positively charged membranes, in which case the filter essentially functions as a physical matrix and removal is charge-based similar to an ion exchange column, and, not size-exclusion based). Regulatory guidelines (CHMP, 1995; FDA, 1998) stipulate that the mechanism of viral clearance must be clearly understood.

The decision with regard to filter type and mode (DFF/TFF) will be governed by the product attributes, that is, the size of the target molecule and protein concentration, its conformation, impurity/particulate load in the feed to be processed; process requirements and limitations (allowable pressure, processing time, batch volumes); characteristics of the potential viral contaminant, that is, virus size (if removal is size-exclusion based), and surface characteristics (if removal is by mechanisms other than size exclusion). Process considerations such as availability of an in-process applicable integrity test and logistics of viral clearance studies, that is, scale-up and scale-down for process evaluation (validation) studies must also be evaluated. For example, large plasma proteins, such as plasma-derived Factor VIII are not filterable through a virus-retentive filter without a significant loss in product recovery. DF filters, require product streams of high purity, to ensure process feasible fluxes. While TF filters can handle a higher particulate load, some TF filters require feedstreams with low protein concentration and the advantage gained by using the TF filter must be balanced against the requirement for process feed dilution (prior to virus filtration) and subsequent concentration post the filtration process.

The available virus filters from different manufacturers can essentially be grouped into two categories: filters designed for "large" virus (>50 nm in size) removal and those that remove the "intermediate" and "small" viruses. The following are the currently available commercial virus filters and the specific claims for the various grades: Asahi-Kasei Medical Corporation: Planova-35N, claims: >5.9 $\log_{10}$ for BVDV (40–70 nm) and >7.3 $\log_{10}$ for HIV; Planova-20N claims: >4.3 $\log_{10}$ for parvovirus (18–26 nm) and >5.4 $\log_{10}$ for EMCV; Planova-15N, >6.2 $\log_{10}$ for parvovirus and >6.7 $\log_{10}$ for

poliovirus (28–30 nm). The claims for the Viresolve filters offered by Millipore are as follows: Viresolve-NFR: >6 $\log_{10}$ for retrovirus (80–130 nm), Viresolve-NFP: >4 $\log_{10}$ for bacteriophage ϕX-174 (28 nm). The available Pall filters and claims are: Ultipor DV50, >6 $\log_{10}$ for bacteriophage PR 772 (76–88 nm), Ultipor DV20, >3 $\log_{10}$ for bacteriophage PP7 (26 nm) and >6 $\log_{10}$ for bacteriophage PR 772. The Sartorius Virosart CPV claims to provide >6 $\log_{10}$ for retrovirus and >4 $\log_{10}$ for bacteriophage PP7. In general, size exclusion-based virus retention is highly reproducible for the "large" viruses and case-specific and less predictable for the intermediate-sized and small viruses.

Considerable ambiguity as regards the nomenclature of virus filters makes selection of virus filters from different manufacturers difficult. For example, manufacturers have assigned ratings based on the particular type of virus retained (e.g., parvovirus, retrovirus), the size of the virus/virus model retained, the molecular weight retention rating achieved based on a surrogate molecular model (e.g., dextran), or on the average pore size established from a mathematical model for the permeability (PDA, 2005). The manufacturer-specified virus filter ratings should be used only as nominal guides, without emphasizing slight difference in manufacturer ratings.

In response to filter-user concerns, a task force comprised of filter manufacturers, industry experts and regulatory representatives addressed the "nomenclature" issue. Virus filters designed for "large virus" removal (i.e., removal of viruses ≥ 50 nm, as for example, HIV, MuLV), - Asahi Planova-35, Pall DV50, Millipore NFR—were evaluated (Brorson et al., 2005). The data demonstrated that these abovementioned filters provided a log reduction value of >6 logs for viruses ≥ 50 nm. Studies to evaluate the performance of the "small virus" removal filters are in progress.

Tandem Filtration

The virus clearance capacity of filters can be augmented by using two filters in series. Troccoli and colleagues (1998) conducted virus-spiked IvIg studies and reported that for EMC (28-30 nm) virus prefiltration of the spiked IvIg through Planova 75N followed by filtration through a single Planova 35N resulted in a total log titer reduction of 3.3 (LTR of 1.1 and 2.2 being provided by the Planova-75 and Planova 35, respectively). Viral clearance could be enhanced by using an additional Planova-35, resulting in a total 4.3 log reduction (for EMC) for this 3 filter system. Similarly, a single Planova-35 nm filter provided 1.7 log reduction for PPV; use of 2 Planova-35 in series resulted in a 2.6 log titer reduction of the challenge virus. In other studies, filtration of virus-spiked Factor IX preparations through a Planova 35 followed by a Planova-15 filter resulted in a LTR of 6.7 and 5.8 for HAV and BVDV, respectively (Johnston et al., 2000).

Protein Transmission

Concomitant with high viral titer reduction is a requirement for good protein transmission. Protein loss may occur either due to protein adsorption to the filter membrane/components or because of protein retention due to either the small effective pore size of the virus filters or so-called "gel polarization" effects. In general, protein loss due to adsorption to the membrane/filter is rarely a problem as many viral retention filters are either inherently hydrophilic (e.g., cuprammonium membranes) or, alternatively, are hydrophilized through surface modifications (e.g., Ultipor VF grade DV filters) to decrease protein binding and enhance product transmission.

If the size of the protein is smaller than the size exclusion cut-off of the membrane, and poor product transmission is observed, other extraneous factors may be responsible. For example, if the product contains significant levels of large macromolecules, protein aggregates, or other particulates, these substances may bind the target protein thus increasing the effective filtration size of the protein and contributing to low product transmission. In such a situation, prefiltration to remove these large particulates may enhance product recovery and flux. Filterability of the product may also be affected by the solution characteristics, that is, pH, ionic strength and other physical parameters, for example, temperature (4°C versus ambient or 35°C). Product loss may also occur within the filtration system hardware and the filter itself; one approach to optimize product recovery is to flush the filtration system with buffer. Note, however, if this is done, scaled down viral clearance studies should be a simulation of the entire filtration process, including the "wash" step. Testing-related non-specific protein binding to the membrane/holder device may also contribute to low recovery in lab scale tests.

Filtration: Process Considerations

From a manufacturing standpoint, in addition to filter performance in terms of consistent virus clearance and high product recovery, a filter user must be able to document that the filter is performing per specifications through a physical test, done during manufacturing that can be correlated to virus retention performance. Whereas a viral challenge is the only true test of a filter's performance, being a destructive test it would preclude subsequent use of the filter in the manufacturing process; additionally, introducing virus into the production facility would be contrary to cGMP requirements. Empirical correlation of the filter function with a measurable physical input parameter is the purview of the filter manufacturer and is often achieved by means of confirmatory installation tests such as integrity test of the filter.

Physical integrity tests for virus filters currently in use include either destructive tests (performed post-use), such as challenge with particulates (e.g., colloidal gold, mixed dextrans) or non-destructive tests (amenable to use both pre- and post- filtration) such as air diffusion tests and liquid porosimetry tests. The filter manufacturer-specified integrity tests and their limitations have been discussed in detail elsewhere (PDA, 2005). While, ideally, the filter should be tested both pre- and post- production filtration, integrity testing of filters post filtration is a regulatory requirement for product release.

Filter manufacturers qualify their viral clearance claims both for flat sheet membranes and for membranes subsequently pleated/cut and sealed into process scale cartridges/cassettes. Virus retention testing is conducted to correlate retention performance of the filter with a physical integrity test applicable under process scale; the "limit values" set by the filter manufacturer usually allow for an "adequate" safety margin. In general, the integrity test results must correlate with the virus removal claims, as specified by the filter manufacturer. Ambiguities exist with regard to what the recommended integrity test detects and it is prudent for the user to query the filter manufacturer. Table 6 provides examples of the types of issues to raise.

VIRUS CLEARANCE EVALUATION (VALIDATION) STUDIES

Viral clearance evaluation (validation) studies are not "validation" studies in the strict sense of the word, validation, as defined in the "Guideline on the General Principles of

TABLE 6 Integrity Test-Related Questions to Raise with the Filter Vendor

- What is the scientific principle on which the integrity test is based?
  - What is the integrity test designed to detect?
  - Is it sensitive enough to detect pinholes? Does it detect gross defect?
- Destructive or non-destructive test?
  - This will determine whether the integrity test can be performed both pre- and post-use
- Is the test correlated to viral retention?
  - If so, which virus?
- Is there a validation guide/other documentation to support it's use in a bio/pharmaceutical environments?
  Query the filter vendor as regards practical considerations
  - integrity test solvent used
  - Process Considerations: test time during actual manufacturing (can the entire operation—product filtration and post-use, in-process integrity test be completed in a single shift
  - Is the test performed in situ or off-line?

Process Validation" (FDA, 1987); nevertheless, virus validation studies is a term commonly used and the terms "virus validation" and "virus clearance evaluation" are used interchangeably in this report.

Due to analytical limitations (discussed earlier in this chapter), it is impossible to demonstrate absolute absence of viral presence. Viral validation studies are, therefore, conducted both to document clearance of viruses known to be associated with the product (e.g., HIV, hepatitis viruses, and parvovirus in the case of plasma products) and also to estimate the robustness of the process to clear potential adventitious viral contaminants (that may have gained access to the product) by characterizing the ability of the process to clear non specific "model" viruses.

Process Analysis and Evaluation of Processes to Validate For Viral Clearance

Ideally, strategic planning for process validation must begin early in product development. The first steps in the validation process involve a critical analysis of the bioprocess to determine likely sources of viral contamination (including pathogenic potential of these contaminants) and process characterization to identify which steps in the manufacturing process have the potential for viral clearance.

Each process step to be tested for viral clearance should be evaluated for the mechanism by which virus clearance occurs, that is, whether it is by inactivation, removal or a combination of both. A "robust" step is one where the viral clearance (inactivation/removal) effectiveness is widely independent of variability in production parameters (Willkommen et al., 1999). Both serendipitous methods (those routinely used in the manufacturing process and which have coincidental viral clearance capability, for example, chromatography and low pH-buffer elution steps) and methods deliberately incorporated for the precise purpose of viral clearance (e.g., filtration and heat inactivation) are usually validated.

Regulatory guidelines (CDER, 1998) recommend the incorporation of multiple orthogonal methods for viral clearance, that is, methods that have independent (unrelated) clearance mechanisms. One misconception is that an entire manufacturing process which may include, for example, ion-exchange chromatography, pH inactivation and detergent inactivation, can be tested by challenging with a large spike of virus during the first step and sampling during subsequent steps. Logistically, this is impossible for two reasons: (*i*) based on the product and possible contaminants, most processes require demonstration of >12–15 logs of clearance for individual viruses, and, it is not possible to grow mammalian viruses to such high concentration; and, (*ii*) using a low viral challenge level will result in an initial low viral load, with each successive step in the bioprocess being challenged with fewer viral particles (assuming the previous steps are effective at inactivation/removal of viruses). This study design would also restrict the number of viral clearance steps that can be claimed and reduce the overall claim that can be established for the entire process. The best compromise is to evaluate each of the individual orthogonal steps separately and then sum the amount of clearance obtained for the entire process. While this method may have some limitations and introduce errors due to overestimation of clearance it is the only practical approach to a complex problem.

Viral Clearance Studies: Scaling Considerations and Identification of Critical Parameters

Typically, virus clearance evaluation studies are conducted at scaledown conditions due to logistic limitations. The scale down must be a true representation of what occurs in the manufacturing process, that is, process modeling must be accurate. Depending on the process, critical operating parameters to be conserved in scaled down studies include: volume, flow rates, contact time and product and/or contaminant load. The composition of the test material should be similar in terms of protein concentration, pH, ionic strength, etc; product generated by the large- and small-scale processes should be similar in terms of purity, potency, and yield. Other process parameters should also be evaluated for possible impact on viral clearance to determine if they should be included in the scaled down study model.

Regulatory guidelines recommend use of virus validation data to set in-process limits in critical process parameters. While validations are usually conducted at both process extremes, viral studies being costly and time consuming, testing at both process extremes is usually not done. Instead, testing is performed under "worst case" conditions to demonstrate the minimum clearance a step can provide. "Worst case" conditions will vary depending on the method and is determined by those factors that influence the clearance mechanism.

"Worst case" Conditions: Virus Removal Methods

In chromatographic processes, depending on the resin and binding mode, critical variables include product/contaminant concentration, buffers, flow rate, wash volumes, temperature, etc. For example, with chromatography in the product binding mode, (depending largely on the resin being evaluated), due to competitive binding for interactive sites, the kinetics of virus binding would be enhanced in the presence of the lowest product concentration (which would constitute "worst case"). Using the minimum wash volume before elution would also encourage virus to elute with the product. As with all chromatographic processes, flow rate will influence kinetics. For chromatography in a contaminant/impurity binding mode, "worst case" contaminant conditions may be

achieved by either increasing the contaminant to product ratio or loading the column with a larger volume of product than is processed during manufacture. This will provide competition between virus and the expected contaminants and impurities. Using the largest post load wash volume expected in the manufacturing process (before the first cleaning step) will remove the maximum amount of virus from the resin along with the product and thus constitute "worst case" (Brorson et al., 2005). In the case of filtration studies, depending on the filtration mode (direct flow/tangential flow), variables include composition of the solution to be filtered (nature of protein, protein concentration, other solution characteristics such as pH and ionic strength), process-associated factors such as differential pressure and flux, and the appropriateness of downscaling, that is, ratio of filter volume to filter area (Aranha, 2001b).

"Worst-Case" Conditions: Inactivation Methods

Any viral inactivation method should result in irreversible loss of viral activity. Viral inactivation kinetics is rarely linear and sometimes a small residual fraction of the viral contaminant resistant to the inactivation strategy may persist. By performing kinetic inactivation experiments involving several time points, the rate of inactivation and thus the potential margin for safety in the actual production process can be assessed.

Variables in inactivation studies include exposure time, temperature, product concentration, presence/absence of contaminant protein, volumes, flow rates and container equivalence. General considerations to be borne in mind are the necessity to ensure sample homogeneity prior to the treatment strategy, use of calibrated equipment (e.g., timers, chart recorders), and equipment qualification.

In the case of pH inactivation studies, low pH inactivation, in general, is considered robust at values of 3.9 or below but may be effective at different ranges for different lengths of time. Choosing a pH value closest to neutral within the range tested will provide a "worst case" challenge as will the shortest time. High protein concentrations, in general, have a protective effect, and, consequently, product (protein) concentration should be maximized (within process ranges) to ensure "worst case" conditions when conducting viral clearance studies.

Variables in the case of detergent inactivation include concentration, exposure time and exposure temperature. Additionally, detergents being viscous, it is imperative to ensure sample homogeneity. The lowest detergent concentration combined with the shortest time provides a "worst case" condition. Temperature could be an important factor and may need to be evaluated at the extremes during development to determine its effect. In general, the lowest temperature provides the slowest kinetics. For heat inactivation studies, temperature distribution must be uniform and timing must begin only when steady state is reached. "Worst case" in heat inactivation studies would constitute the highest stabilizer concentration used, the highest product concentration, and the lowest temperature. If scaled down studies are conducted, container equivalence must be demonstrated. Appropriately calibrated equipment, for example, timers, chart recorders must be used; equipment qualification is mandatory.

Viral Clearance Evaluation (Validation) Studies: Virus Filtration-related Issues

Any viral clearance process operational during manufacturing must be demonstrated to be effective under "worst case" conditions. Filter users conduct viral clearance evaluation (validation) studies often under considerably "scaled-down" conditions (100–1000× downscaled) and, therefore, process modeling must be accurate. Critical parameters will

vary depending on the unit operation being evaluated. For example, in the case of filtration, filtration mode (direct flow/tangential flow), filtration-associated factors (flux, transmembrane pressure), and, product-related considerations including protein concentration and solution characteristics (pH, ionic strength) must be considered. Experimental design-associated variables will also considerably impact clearance results and the potential for erroneous clearance factors from this source must also be recognized. Virus-spike related considerations have notably been one of the key factors contributing to differences in test results; another factor is appropriateness of the process modeling in terms of downscaling, ie, ratio of product volume to filter area. Testing process devices such as the geometry of the filter housing and the pumping device (peristaltic/gear pump, etc.) that may generate shear forces and result in viral inactivation (rather than removal) may be another contributing factor.

Viral challenges must be conducted with viral spikes that represent a "worst-case" challenge. In filtration studies, viral aggregation either due to the method of viral stock preparation, virus attachment to membrane particulates, or virus binding to proteins/other antibodies in the product, will enhance the retentive capacity of the filter and will provide false clearance values. One approach to reduce viral aggregates is to prefilter the viral-spiked product; prefiltration through "sterilizing grade" filters (0.2 or 0.1-μm-rated) or through virus removal filters (grade DV50, Planova-35) has been used.

Product load is another variable in clearance evaluation studies. In direct flow filtration, "worst case" would represent a combination of the highest mass to surface area ratio and highest product (protein) concentration. In TFF for viral clearance, "worst case" conditions include the lowest product concentration expected and the highest number of diafiltration volumes to be used. In TFF applications, high protein concentrations are conducive to formation of a gel polarization layer on the filter surface and this will enhance virus retention. Enhanced retentivity as a consequence of membrane gel polarization may provide acceptable results in bench scale tests but these effects are difficult to model reproducibly and removal due to process-related conditions is not considered "robust". Product solution characteristics, that is, pH, ionic strength, are another factor that can influence virus retention.

Virus aggregation, either as a consequence of the nature of the suspending medium or due to specific binding (antigen-antibody reactions, as in the case of plasma products that may contain neutralizing antibody) can enhance the observed titer reduction. For example, parvovirus B19 was efficiently eliminated by filters having nominal pore sizes larger than the diameter of the respective free virions (Omar and Kempf, 2002). Burnouf-Radosevich et al. (1994) reported removal of large infectious doses of bovine parvovirus from highly-purified factor IX and XI concentrates, which they attributed to virus aggregation (Burnouf-Radosevich et al., 1994). Yokoyama and colleagues demonstrated EMCV, parvovirus B19, and PPV aggregation in the presence of certain amino acids, which resulted in their removal by filters with pore size larger than the size of the viruses (Yokoyam et al., 2004).

In general, some common reasons for ambiguous and erroneous data with "virus-filter" evaluation studies and failure at manufacturing scale include: inaccurate process modeling (inappropriate scaledown), enhanced viral retention due to gel layer/particulates that contribute to false log reduction values, membrane fouling (and inability to get scaled down volume through the small scale device) due to additional protein load (stabilizers/serum/particulates) in virus spike, and, exceeding manufacturers recommended operating conditions in the case of filtration studies. These variables must be borne in mind when evaluating filters for virus removal and designing viral clearance evaluation studies.

Technical Aspects of Study Design

Choice of panel of test viruses. There is no single indicator species to be employed for virus validations. Choice of the appropriate panel of viruses to use will depend on the source material (plasma-derived biologicals versus cell-line derived) and on the product phase at which viral clearance testing is conducted.

In general, the panel of test viruses used should include relevant viruses (i.e., known/suspected viral contaminants), and model viruses. Relevant viruses are, for example, HIV and hepatitis B and C viruses, which are known contaminants of blood products. Some relevant viruses, for exampel, Hepatitis B and C viruses are difficult to propagate *in vitro*; in these cases specific model viruses may be used. Specific model viruses are viruses that resemble known viral contaminants; for example, BVDV and Sindbis virus have been used as models for Hepatitis C virus. Similarly, murine leukemia virus (MuLV) is often used as a model for non-infectious endogenous retroviruses associated with rodent cell lines. Additionally, non-specific model viruses are also included in the test panel to characterize the theoretical clearance capability of the manufacturing process, that is, assess the "robustness" of the process. These include viruses of different size and varied physicochemical and biophysical characteristics; they are not expected to be associated with the product but are included to address theoretical safety concerns and add confidence that the process can handle unknown or undetected viruses. Examples of viruses that have been used in virus validation studies are provided in Table 7.

In some cases, in view of the cost-prohibitiveness of an entire virus validation package, preliminary testing with surrogates such as bacteriophages can be undertaken. Such testing is, of course, relevant only if removal is size-based, as in filtration; if clearance is dependent on a particular physicochemical or other surface characteristic of

TABLE 7 Test Viruses Commonly Used in Viral Clearance Evaluation Studies

Virus	Family (-viridae)	Genome	Envelope	Size (nm)	Shape
Vesicular stomatitis virus	Rhabdo-	RNA	Yes	70 × 175	Bullet
Parainfluenza virus	Paramyxo-	RNA	Yes	100–200 nm+	Pleomorphic/ Spherical
Pseudorabies virus	Herpes	DNA	Yes	120–200	Spherical
Herpes simplex virus	Herpes	DNA	Yes	120–200	Spherical
Human immunodeficiency virus (HIV)	Retro	RNA	Yes	80–100	Spherical
Murine Leukemia virus (MuLV)	Retro	RNA	Yes	80–110	Spherical
Reovirus 3	Reo	RNA	No	60–80	Spherical
Sindbis virus	Toga	RNA	Yes	60–70	Spherical
SV40	Papova	DNA	No	40–50	Icosahedral
Bovine viral diarrhea virus (BVDV)	Toga	RNA	Yes	50–70	Pleomorphic/ Spherical
Encephalomyo-carditis virus	Picorna	RNA	No	25–30	Icosahedral
Poliovirus	Picorna	RNA	No	25–30	Icosahedral
Hepatits A	Picorna	RNA	No	25–30	Icosahedral
Parvovirus (canine, murine porcine)	Parvo	DNA	No	18–24	Icosahedral

the virus, it cannot be used. The applicability of bacteriophages as surrogates for mammalian viruses in filter validation studies has been addressed elsewhere (Aranha-Creado and Brandwein, 1999). While data corroborating similar retention performance with both mammalian viruses and appropriately sized bacteriophages has been available for some time, only recently has a formal assessment of their comparability been attempted. A recent task force report (PDA, 2005) on the initiatives towards virus filter nomenclature standardization for "large pore size" virus filters provided the necessary impetus to study this issue. Bacteriophage PR 772 has been demonstrated to be an appropriately model for evaluation of "large pore size" virus retentive filters (Lute et al., 2004) and performance of these filters from several virus filter manufacturers has been evaluated and demonstrated to be comparable (Brorson et al., 2005). While virus clearance data for regulatory submissions currently must be obtained with mammalian model viruses, the demonstration of the comparability of retention of appropriately sized bacteriophages and mammalian viruses provides a rationale for the use of bacteriophages in process development work prior to a regulatory submission.

Virus-stock related considerations. The quality of the stock preparation and the titer of the virus spike will significantly influence the test results and the ability to make a viral clearance claim. To-date, there are no standardized methodologies for preparation and purification of virus stocks; this matter is a subject of concern and is currently being addressed by a PDA/FDA study group.

In general, starting with a high viral load to challenge a process step will maximize the potential viral clearance claim. The volume of virus spiked into the challenge material and the virus stock titer combine to determine the total virus titer in the spiked product. The virus density depends primarily on the biology of the virus and can vary from virus to virus. While it is advisable to work with high titer virus stocks, keep in mind that methods used to concentrate the virus stock and achieve high stock titers may be conducive to virus aggregation.

The quality of the virus stocks in terms of presence of viral aggregates, cell debris or other particulates can influence the results by causing a false enhancement or reduction of viral clearance. Thus, for example, with a chromatography process in a contaminant binding mode, extra cell debris may compete with the virus for binding sites on the resin causing a decreased clearance value. In a tangential flow filtration process, use of a virus stock containing high amounts of cell debris would enhance virus retention due to the polarization of the membrane. In direct flow filtration, if the membrane clogs prematurely due to cell debris the entire load volume cannot be filtered and, therefore, the full log clearance cannot be claimed.

Viral spike volumes will impact clearance studies (especially if there are large amounts of debris), and, in general, should be maintained at 10% or less of the final volume to keep the feedstream representative of the manufacturing process (CHMP, 1995).

Importance of adequate controls in virus study design. The importance of controls cannot be overemphasized. Controls allow for attributing clearance effects to actual treatment procedures versus artifacts of the test design and methodology. The following are some common controls to be included for virus clearance evaluations.

Prior to the viral clearance assays, it is necessary to ascertain that the product does not have an inhibitory effect either on the indicator cell line (generalized cytotoxicity control) or the test virus (viral interference studies). Cytotoxicity and interference controls are often conducted considerably in advance of the actual validation study to ensure that the clearance capacity is not overestimated due to test-related considerations. The cytotoxicity control is included to ensure that any indicator cell cytopathology observed during the study is due to the virus alone. The cells are exposed to process components (product intermediates, buffer), in the absence of virus for the length of time

the test material will be in contact with the cells; a cytopathic or morphological effect relative to the unexposed control cells is an indication of cytotoxicity. Viral interference studies are conducted to determine if process components interfere with the capacity of the test virus to infect the indicator cell line. Essentially, following exposure of the indicator cell line to the process component, the cells are exposed to the virus and evaluated to determine if there is any loss of infectivity and thus viral interference by the product. If either of the above two controls demonstrate positive results, one approach is dilution of the test material (in order to determine a non-inhibitory concentration); another is neutralization or other test solution adjustment if needed.

The hold control is included to ensure that the test virus is stable throughout the test duration in the presence of test material and essentially involves virus-spiked starting material held for process time at process temperature. This control essentially demonstrates any inactivation effect that is a consequence of the product (start material). The loss demonstrated by a hold control is not related to the clearance strategy under study and should be evaluated accordingly.

Stability and storage issues are primarily a concern if process challenges are performed at a site different from that of the virus vendor. If virus stocks are to be shipped to another location, the stocks are thawed, processed over the manufacturing step to be challenged and frozen for later shipping. This may differ from challenges performed at the vendor site in that many vendors often assay the test material immediately. This reduction may affect the final clearance claim; freeze/thaw stability should be reviewed

"Shipping" controls determine if temperature changes that may have occurred during shipping affected titers when virus is shipped to a different site.

Viral Clearance Validation Studies: Pitfalls and Cautions

As discussed earlier, a "good" viral clearance validation study is the consequence of a detailed and well-designed study. Scaled down studies are, at best, approximations of conditions achieved under manufacturing conditions and the validity of the clearance data is a direct reflection of accurate process modeling and study design. Some of the pitfalls associated with small scale validation studies are related to the following: (*i*) virus-related considerations. Viral spike-related perturbations may make the process non-representative of actual manufacturing conditions. Also, model viruses are used in process validation studies; these are, at best, just that—models - and the wild type strain may not behave similar to a laboratory strain. (*ii*) Inaccurate process modeling. Conditions in small scale validations may not always be congruent with process scale conditions, for example, columns used only once for a validation may not reflect the ability of columns used repeatedly (during manufacture) to remove virus consistently; certain sites on the resins may become blocked on repeated use, reducing the effectiveness of virus removal over the resin lifetime. (*iii*) Sample-related considerations include non-representative sample used in viral validations, for example, either the proper intermediate or actual product sample may not have been used; sample may not be representative in terms of protein concentration, pH or other solution characteristics such as ionic strength; samples may be non-homogenous due to inadequate mixing. (*iv*) Assay-related considerations include failure to evaluate buffer toxicity, poor model virus selection, lack of appropriate controls and poor standardization of viral assays. Critical assay performance criteria are accuracy, reproducibility, repeatability, linearity of range, limit of detection and limit of quantification and must be validated (Darling et al., 1998).

Steps that require dilution of the product (e.g., due to viral interference or other toxicity-related considerations) will impact assay results and the ability to make a high viral clearance claim. For example, high salt concentration, pH extremes or other sample conditions may interfere with the virus titration. Decreasing the actual volume assayed (due to dilution of the sample, e.g., 10×) will result in decreased sensitivity and is especially important when no virus is detected and a theoretical limit titer for the sample is calculated.

In general, overall greater reduction factors can be claimed with a larger number of observations from larger volumes performed with tests with the lowest available limits of detection when complete clearance for a step is expected. Virus quantitation methods can be modified to enhance sensitivity by use of additional replicates and increased inoculation volumes. Large volume assessment can be used as a supplement to conventional titration methods to increase the probability of detection for extremely low virus concentrations.

Considerations in Data Interpretation and Estimating Viral Clearance

Establishing clearance for the entire process (overall clearance value) requires at least two orthogonal "robust" methods of viral clearance. The individual steps must possess fundamentally different mechanisms of virus removal or inactivation in order for values to be considered cumulative. Only data for the same model virus is cumulative since viruses vary greatly with regard to their inactivation or removal profiles. Clearance estimates and their variances are calculated for each orthogonal unit operation; total virus reduction is the sum of individual log reduction factors. In cases of complete clearance, a theoretical titer value is based on a statistical distribution (Poisson distribution). Table 8 provides cumulative virus clearance values for Murine Leukemia Virus.

The clearance goal is usually chosen based on the product use and the risk to the patient population. The extent of product testing necessary will depend on source and nature of the product, the stage of product development and the clinical indication. Abbreviated testing may apply in serious or immediately life-threatening conditions for which no effective alternative treatment exists.

TRANSMISSIBLE SPONGIFROM ENCEPHALOPATHY AGENTS

Animal and human-derived materials find applications in biologicals and therapeutic products manufacturing, novel tissue-engineered products, and xenotransplantation.

TABLE 8 Example of Calculation of Viral Clearance (Log Titer Reduction) for the Overall Process for a Monoclonal Antibody Product

	Log reduction factor
Ion exchange chromatography	> 5.39
Nanofiltration	> 5.14
Low pH inactivation	> 5.85
Detergent inactivation	> 6.06
Total clearance	> 22.44

Note: Challenge virus: Xenotropic Murine Leukemia virus (X-MuLV).
Source: Aranha and Forbes, 2001.

Bovine-derived products are used in the medical devices and biopharmaceutical areas, as well as the cosmetics and food industries, either as active ingredients or as raw materials during production of a biopharmaceutical. Tables 9 and 10 provide further details.

Transmissible spongiform encephalopathy (TSE) is a generic term used to describe progressive neurodegenerative diseases caused by unusual/novel infectious agents. The bovine spongiform encephalopathy (BSE) epidemic ("mad cow disease") in the mid eighties raised public awareness to the manifestation of these diseases, collectively referred to as TSEs. These diseases are invariably fatal and are characterized by a long incubation period, and a short clinical course of neurological signs. Creutzfeldt-Jakob disease (CJD) is one of the human manifestations of TSE. It may be sporadic (arise spontaneously at low frequency), be acquired from exogenous exposure (especially iatrogenic sources such as via contaminated surgical instruments, dura mater implants, corneal grafts and cadaveric pituitary-derived hormones) or familial (due to mutations in a gene on chromosome 20). TSE disease manifestations in humans and animals are tabulated in Table 11.

TABLE 9 Uses of Bovine-Dervived Constituents in Industry

Pharmaceutical/BioPharmaceutical Industry
Serum albumin as an excipient, protein supplement, universal reagent
Serum proteins as carriers for water-insoluble components
Aprotinin in cell culture, protein purification processes
Gelatin for plasma-derived applications
Trypsin in cell culture applications
Fetal calf serum/Newborn calf serum as a growth additive in cell cultures
Glycerin and glycerin derivatives in cough syrups, external analgesics, topical antibiotic preparations, several over-the-counter products
Medical/Medical Device Industry
Bovine pericardium used in heart valves
Bovine collagen in lacrimal plugs, other surgical applications such as tissue sealants, artificial skin
Injectable collagen in plastic surgery
Bovine hyaluronidase for cartilage and joint treatment
Bovine heparin as a blood thinner
Cosmetics Industry
Glycerin derivatives in aftershaves, beauty products, hair care products
Gelatin in lotions/creams, shampoo bases, protective creams
Fatty acids used in cosmetics and soaps
Food Industry
Gelatin in confectionaries, jams/jellies, dairy products such as yogurts, icecreams and sorbets
Gelatin in nutraceuticals and other food additives
Gelatin used as a fining agent for the clarification of wines
Edible tallow used in shortening for baked goods and chewing gum
Agriculture
Bovine proteins as fertilizer
Industrial Applications
Bovine proteins in adhesives, in dye industry
Bovine gelatin for photographic uses
Bile acids to make industrial detergents
Fatty acids from tallows used in the plastics, rubber, paint, automotive, detergent industry

TABLE 10 Categorization of Bovine Materials used in the Manufacture of Medicinal Products

Use	Examples
Active Ingredients	Glucagon (derived from bone pancreas)
	Aprotinin (dertived from lung of bovines and other animals)
	Heparin
	Collagen
	Amino acids
Inactive ingredients/ excipients	Gelatin
	Amino acids
	Tallow derivatives
Raw materials, reagents used in manufacture	Bovine serum albumin
	Bovine serum (fetal bovine serum, fetal calf serum, donor serum)
	Enzymes
	Culture media
	Tallow derivatives, e.g., Polysorbate-80 used in purification processes, e.g., viral inactivation step using solvent-detergent treatment

A new variant form of CJD (vCJD), that differs from classical human CJD and believed to have originated from BSE was first reported in 1996 (Will et al., 1996). Several lines of experimental evidence (Bruce et al., 1997; Collinge et al., 1996; Hill et al., 1997; Lasmezas et al., 1996; Farshid et al., 2005) indicate that the causative agents for BSE and vCJD are indistinguishable (Aguzzi and Miele, 2004; Aguzzi and Weissmann, 1997), which raises a serious concern of inter-species transmission from bovines to humans via ingestion of contaminated meats. This inter-species transmission could result in intra-species amplification via exposure to iatrogenic manipulations (blood transfusions, organ transplants, etc.). This has significant implications from a public health standpoint. The reported differences between classical (sporadic) CJD and vCJD are tabulated in Table 12.

The causative agent of TSEs has yet to be conclusively demonstrated. Agents implicated in the etiology of TSEs include an infectious protein (prion theory); an unconventional/slow virus or a bacterium; also, an autoimmune etiology, where contaminant bacteria/bacterial fragments present in animal feeds could have resulted in molecular mimicry between bacterial components and bovine tissues, has been proposed. The current consensus of opinion is that the etiological agent is devoid of informational nucleic acid and is a protein (prion—proteinaceous infectious agent).

There is no definitive data on the nature of prions. The term "prion" is used as an operational term for the TSE agent (Auzzi and Weissmann, 1997). The conversion of a ubiquitous cellular prion protein (PrP^{c}or PrPsen) to the pathology-associated isoform (PrPsc or PrPres) is one of the hallmarks of TSEs. While "PrPsc" was used to refer to the abnormal PrP isoform, the more generic PrPTSE is the preferred terminology as the "sc" in PrPsc refers to the sheep disease scrapie and is inappropriate when referring to TSE diseases other than scrapie. Nomenclature used for the prion protein isoforms is presented in Table 13.

Transmissibility of infectivity may be influenced by several factors including the route of administration, dosage, the strain of agent used, and the presence/absence of a species barrier. Distribution of infectivity in tissues varies; for example, neural tissue is considered high-risk whereas secretions such as saliva and milk are considered as

TABLE 11 Transmissible Spongiform Encephalopathies in Humans and Animals

Disease (susceptible species)	Transmission/mechanism of pathogenesis
Animal Diseases	
Bovine spongiform encephalopathy (cattle)	Oral; via ingestion of contaminated MBM
Scrapie (sheep)	Infection in genetically susceptible sheep
Transmissible mink encephalopathy	Infection with prions from sheep or cattle
Feline spongiform encephalopathy (cats)	Ingestion of contaminated MBM
Chronic wasting disease (mule deer, elk)	Unknown
Exotic ungulate encephalopathy (greater kudu, nyala, oryx)	Ingestion of contaminated MBM
Human Diseases	
Creutzfeldt-Jakob disease (CJD)	
Sporadic	Somatic mutation in PRNP gene or spontaneous conversion of PrP^c to PrPres
Iatrogenic	Infection via contaminated equipment (EEG electrodes), tissue (corneal) and organ (dura mater) implants, hormone administration (gonadotropin, growth hormone)
Familial	Germline mutation in PRNP gene
Variant	Oral; ingestion of bovine prions in contaminated beef
Kuru	Oral; through ritualistic cannibalism
Fatal familial insomnia	Germline mutation in PRNP gene
Gerstmann-Straussler-Scheinker (GSS) disease	Germline mutation in PRNP gene

"Category C" tissues—tissues with no detectable infectivity (Table 14). Intravenous administration is believed to result in a 10-fold reduction in infectivity compared to the intracerebral route, while a species barrier may result in up to 1000-fold reduction in infectivity (Bader et al., 1998). The incubation period varies depending on the route of exposure; for example, in cases of iatrogenic transmission of CJD via parenteral transmission, symptoms did not develop until several years (~12 years) after exposure; however, post dura mater grafts and neurosurgical procedures the incubation period was months rather than years.

PrPres is the only specific biochemical marker of prion diseases and is used as a surrogate marker for infectivity. PrPres' insolubility in nonionic detergents (Bolton et al., 2005; Meyer et al., 1999) and its partial resistance to proteinase K (Prusiner et al., 1981) allow for distinguishing it from its cellular counterpart (PrP^c). Immunoassays for PrP detection include Western blots, dot blots and ELISA systems (Grathwohl et al., 1997; Lee et al., 2000).

PRION CLEARANCE: A RISK-BASED APPROACH

A precise risk assessment cannot be made with human TSEs due to gaps in our information. Significant unknowns include the etiological agent *per se,* the minimal infectious dose, the exact distribution of infectivity in tissues and uncertainties in key epidemiological parameters.

TABLE 12 Main Characteristics of Sporadic and Variant CJD Disease

	Sporadic CJD	VCJD
Incidence	~1 case/million population/year	165 cases globally[a]
Distribution	Worldwide	EU (UK primarily, France, Ireland)
Age at onset (mean)	Late middle age; 55–70 years; avg ~60 years; occasionally affects younger people	Relatively young age of onset; median ~28 years; range 19–39
Presenting features	Mental deterioration (dementia, myoclonus)	Behavioral abnormalities, ataxia, dysaesthesia Psychiatric and/or sensory symptoms
Clinical course	Rapidly progressive	Insidious onset; prolonged course
Duration (mean)	Short; ~7 months	Long; ~14 months(range 7–38 months)
EEG	Typical periodic pattern	Non-specific
PRNP genotype (codon 129)	Predominantly Met/Met homozygous	100% of the cases have Met/Met homozygosity
Neuropathologic features	Synaptic deposits; rarely plaques	Prominent "florid" plaques
PrP-res banding pattern	Types 1 and 2[b]	Type 4 (similar to experimental BSE in mice, macaques, and other species)
PrP Positivity in tonsil/spleen/ lymph node	No/No/No	Yes/Yes/Yes Presence of PrP in lymphoreticular tissue

[a]Total number of definite or probable cases (dead and alive) as reported by the National CJD Surveillance Unit, Edinburgh, as of Janyary 22, 2007.
[b]Type 3 is found in iatrogenic cases with intramuscular inoculation

Risk assessment to determine the potential for prion contamination of the biological includes evaluation of the source of raw materials, the type of tissue used, and the route of administration. Also, the potential risk should be evaluated in the context of its use. For example, bovine neural tissue implanted in the central nervous system would pose a much greater risk than a few microliters of highly purified bovine pancreatic trypsin used in a manufacturing process to recover tissue culture cells which are further purified before use.

Considerable emphasis is placed on the source of raw material and type of tissue used. For example, certain tissues and body fluids constitute a high risk (e.g., brain, spinal cord) whereas others have no demonstrable infectivity (saliva, skeletal muscle) (WHO, 1999). Note that while blood-associated infectivity was considered questionable a few

TABLE 13 Nomenclature for Prion Protein Isoforms of the Prion Protein

PrPc	Normal cellular isoform of the prion protein
PrPsc	Abnormally folded pathogenic isoform (named after the sheep disease scrapie
PrPres	Protease-resistant prion protein, a property of PrPsc
PrP27-30	The major fragments of PrP remaining intact after digestion of the N-terminus with proteinase K (refers to their mass in kDa)
PrPTSE	Currently used generically, to replace PrPsc and refer to TSE diseases as a whole

TABLE 14 Distribution of Infectivity in Tissues

Category	Description	Examples
Category A high infectivity tissues	Central nervous system (CNS) tissues that attain a high titer of infectivity in the later stages of all TSEs, and certain tissues that are anatomically associated with the CNS	brain, spinal cord, eye
Category B lower-infectivity tissues	Peripheral tissues that have tested positive for infectivity and/or PrPsc in at least one form of TSE	CSF, kidney, liver, lung, lymph nodes/spleen, placenta
Category C tissues with no detectable infectivity	Tissues that have been examined for infectivity without any infectivity detected and/or PrPsc, with negative results	adipose tissue, adrenal gland, gingival tissue, heart muscle, intestine, peripheral nerve, prostate, skeletal muscle, testis, thyroid gland, tears. nasal mucous, saliva, sweat, serous exudates, milk, semen urine, faeces, blood

Source: WHO, (2000).

years ago, blood transfusion-associated vCJD transmission has been recently confirmed (2 cases) and this raises the concern of blood being a vehicle for iatrogenic transmission of vCJD. As Brown (2004) expounds: "whatever the right term ... –possible, probable, or highly unlikely—it has taken the word "hypothetical" out of the human blood infectivity vocabulary". He inverts a well-known aphorism and states "where there is fire (infectivity) there is almost certainly smoke (PrPres)".

Risk Minimization

Risk minimization strategies have included active surveillance strategies, emphasis on appropriate sourcing and import restrictions, severe restrictions on specified bovine and ovine offals entering the human food chain, and a ban on feeding of mammalian proteins to ruminants, and on cattle over 36 months of age entering the human food chain. Further measures in the United Kingdom to minimize the risks of establishing a reservoir of human-to-human infectivity include a ban on the use of UK-sourced plasma for the preparation of licensed blood products (e.g., coagulation factors, albumin), leukodepletion of blood donations, increased levels of decontamination of surgical instruments and use of disposables wherever possible.

Sourcing is the cornerstone of the risk minimization initiative. For biopharmaceuticals using bovine-derived materials, species, and source country of the bovine-derived constituent must be identified. A geographic bovine risk (GBR) 4-category classification of countries or zones with regard to BSE status has been established (Table 15).

Specific risk minimization approaches apply to production systems. For example, for vaccines grown in animals or those for which bovine-derived materials are used for production sourcing must be from countries with low GBR risk due to the higher risk. On the other hand, master seeds (MCs) or cell banks (MCBs) that have been prepared 30–40 years ago for vaccine production do not need to be reestablished as this would pose a higher risk; for example, there is a potential of altering the vaccines through

rederivatization. CBER has required licensed vaccine manufacturers to evaluate all bovine sourced materials used in fermentation/routine production and in the establishment of working seeds and WCBs to ensure full compliance with the TSE guideline (CPMP/CVMP, 2001). Table 16 presents this information.

Risk Management

In view of the considerable uncertainties and significant gaps in knowledge, risk management programs pose a significant problem. While even a minimal risk could possibly be considered unacceptable in the case of TSEs, risk assessment and management must be evaluated in perspective. Our quandary with the TSEs, and especially with vCJD lies in the fact that as TSEs are relatively rare neurological disorders, risk assessments are hampered by a limited statistical power. Additionally, uncertainties in key epidemiological parameters make quantitative risk assessment difficult. Specific preventative and surveillance actions taken since BSE was first diagnosed in the United Kingdom in 1986 are presented elsewhere (Aranha, 2005).

Prion Clearance Methods

Prions are notoriously hardy to methods that would be considered overkill for most other microbial and viral agents. Part of this resilience could be attributed to its propensity to be associated with organic matter/cell debris that confers a protective effect; another contributing factor is its preponderance to form aggregates (Masel and Jansen, 2001).

Prion clearance methods used should essentially be reliable, robust and preserve product integrity. This becomes a challenge especially in the face of an agent whose significant hardiness to inactivation conditions has been consistently demonstrated. In view of the high resistance of prions and the lability of biopharmaceuticals, removal methods such as filtration, precipitation, and chromatography appear to be more applicable than inactivation methods.

There is no single method that has been shown to be 100% effective to inactivate prions. In general, TSE agents have high resistance to a variety of physical and chemical treatment methods: dry heat (160°C, 24 h) has no effect; infectivity remains even after exposure to 360°C, 1 h, autoclaving (infectivity detected post autoclaving at 126°C, 1h), UV and ionizing radiation (Bellinger-Kawahara et al., 1987; Taylor and Diprose, 1996), alcohols and alkylating agents, phenolic disinfectants, etc. (Brown et al., 1990, 1982; Dickinson and Taylor, 1978; Ernst and Race, 1993). β-propiolactone is also reportedly ineffective (Haig and Clarke, 1968).

Caution must be exercised when reviewing the literature pertaining to prion inactivation as the studies vary in the prion strains used (CJD, scrapie, BSE), kinds of

TABLE 15 European Commission SSC Classification for Geographical BSE Risk (GBR)

GBR level	Presence of one or more cattle clinically or pre-clinically infected with BSE in a geographical region/country
I	Highly unlikely
II	Unlikely but not excluded
III	Likely but not confirmed or confirmed at a lower level
IV	Confirmed at a higher level (≥ 100 cases/1 million adult cattle per year)

Source: EMEA/310/01 Rev 2 – October 2003.

TABLE 16 Risk Minimization Approaches Applied to Production Systems

Production System	Risk elimination/minimization approaches
Vaccines grown in animals	Source animals must be from countries with low GBRmaterials, and, in some cases, postmortem testing of each production animal can greatly reduce the TSE risk testing of each production animal Bovine-derived materials used in the routine production of vaccines that are sourced from countries on the USDA list should be replaced with bovine-derived materials from countries not on the USDA list.
Vaccines cultivated in embryonated eggs	Production system is not "TSE-relevant", so contamination with TSE agents is highly unlikely
Banked eukaryotic or bacterial cells and viral vaccine seeds	Banked cells and seeds undergo extensive characterization, therefore, overall risk-benefit assessment favors their use compared with re-derivatization of banked production systems; also, the biological phenotype of the vaccine may be difficult to recreate and the risk of altering the bacterial or viral vaccine through re-derivation is significant Re-derive working bacterial and viral seed banks and working cell banks that were established using bovine-derived materials sourced from countries on the USDA list using bovine-derived materials from countries not on the USDA list.

different brain preparation methods (dried/macerated preparations, unspun 20% homogenates, 10% tissue supernates, ultracentrifuged material), and kinds of test systems (animal models). Other variables include exposure times, temperature and the type of tissue studied (brain versus reticuloendothelial). Furthermore, any interpretation of experimental data relating to the infectivity or transmission of TSE must be tempered by the acknowledgement that studies are done under experimentally-induced conditions by intracranial injection (high efficiency of the infectious route), of contaminated brain (high titer of infectivity) into non-human primates (low species barrier) to provide accelerated disease progression and prognosis. Often, the assumption is made those procedures effective for partially purified TSE infectivity is equally applicable for dealing with crude tissue contamination, but this is unwarranted (Taylor, 1986).

Bioseparations techniques often include protein precipitation based on differential solubility, and adsorptive behavior. Operations such as precipitation, adsorption and filtration, are commonly used in the manufacture of biopharmaceuticals and plasma-derived biologicals. TSE agents have unique physicochemical properties that make possible serendipitous reduction during processing of biological products. For example, PrPres is readily precipitated by ethanol, ammonium sulfate, and polyethylene glycol. The first step in the manufacture of plasma products involves cryoprecipitation followed by solvent precipitation steps. Despite differences in experimental methods with regard to the kind of spike used (crude brain homogenate versus microsomal fraction), and detection methods (bioassay versus Western blot), relatively consistent clearance factors have been reported by several investigators (Foster et al., 2000; Lee et al., 2001). This suggests that the effects of cold ethanol on PrPsc are relatively robust.

The prion protein is most commonly membrane-associated and has a tendency to aggregate. These attributes facilitate their removal by depth filtration (Foster et al., 2000),

also virus removal filters have been demonstrated to provide prion clearance; chromatographic purification in the manufacture of coagulation factor concentrates is extensively used and these methods have the potential to effect prion removal (Foster et al., 2000).

PROCESS CLEARANCE EVALUATION: CONSIDERATIONS AND DESIGN ISSUES

As in the case of ensuring viral safety of biologicals, a multifaceted approach involving adequate sourcing, incorporation of multiple orthogonal clearance strategies and process evaluation for prion clearance are vital. While there is no specified requirement for demonstration of prion clearance in the case of biotech products, if the product labeling includes any reference to the safety of the product from a prion standpoint, the claims must be substantiated with experimental data.

Currently, there are no in vitro tests directly applicable for detection of low levels of infectivity either in the raw materials or the finished product; consequently, adequate sourcing and demonstration of the prion clearance ability of the manufacturing process constitutes a key paradigm to ensure safety.

The first steps in the design of the clearance evaluation study involve a critical analysis of the entire manufacturing process to determine the potential sources of prion contamination followed by process characterization to evaluate which steps in the manufacturing process possess clearance capability. The principles applied in evaluation of clearance of conventional viruses are applicable to TSE agents (Hellman and Asher, 2000). CPMP (Committee for Proprietary Medicinal Products) guidance documents acknowledge that several routine processing steps such as precipitation, chromatography and nanofiltration can contribute to TSE agent removal and require that whenever TSE clearance claims are made for a particular step, the process should be validated (CPMP, 1996; EMEA, 2000).

A number of studies have been undertaken to evaluate purification process steps for their potential for prion clearance using either a crude brain homogenate (Bader et al., 1998; Lee et al., 2000; Lee et al., 2001), detergent-solubilized (Pocchiari et al., 1991) or a microsomal fraction (Forster et al., 2000) of the hamster-adapted sheep scrapie agent. The kind of spike used will depend on the process step being evaluated.

Issues to address when designing a validation study to document prion removal are choice of spiking agent, nature or form of the spiking agent (and its relevance), the design of the study (including appropriate scale down) and the detection method (in vitro or in vivo assay). Validations are often performed using strains of the sheep scrapie agent that have been adapted to either mice or hamsters, by direct intracerebral inoculation of infected sheep brain into mice or hamsters followed by serial passage of the agent in the same species.

Spiking Agent(s)

Experimental TSE studies have been conducted with a variety of TSE agent spikes—mouse/hamster passaged scrapie agent, mouse-passaged BSE, mouse/hamster/guinea pig-passaged CJD. Process clearance evaluation studies are often performed using strains of rodent-adapted sheep scrapie agent; these models have been accepted by regulatory authorities (Bader et al., 1998).

In general, the hamster-adapted 263 K strain of scrapie is regarded as the optimal system because high titers in the brain combined with a short incubation period are a hallmark of this system. Spike-related considerations include the “relevance” of the spike

and the concentration of the spiking agent. To date, there is no agreement as regards whether there is a minimum threshold concentration required for infection, and, routinely, process clearance evaluation studies are done using high titer spikes to expedite testing.

Detergent-solubilized scrapie agent is often used in filtration studies (detergent treatment of the crude brain homogenate, which is comprised of membrane-bound PrP, results in dissolution of the lipid membrane), as it constitutes a "worst case" challenge. TSE agents in blood would likely be cell-associated; consequently, a more representative spike for plasma products may be a microsomal fraction prepared with crude brain homogenate.

Detection Methods used in Process Clearance Evaluation Studies

Sample analysis is often undertaken either by infectivity assays (Lee et al., 2000; Lee et al., 2001; Brown et al., 1998) or by the Western blot method (Foster et al., 2000; Lee et al., 2000, 2001). While the infectivity assay is the assay of choice, the extended incubation period and the cost-prohibitiveness of the assay present logistic limitations. Though less sensitive than a bioasssay, the Western blot provides a rapid and useful tool for detection and preliminary screening of manufacturing methods for prion clearance.

Considerations in Data Interpretation and Estimating Prion Clearance

Estimating prion clearance for the entire process (overall clearance value) is calculated in a similar manner to viral clearance. Studies in the literature have documented (Golker et al., 1996; Peano et al., 2000) that significant prion clearance can be achieved by both serendipitous and deliberate steps. Clearance estimates and their variances are calculated for each orthogonal unit operation; total reduction is the sum of individual log reduction factors.

The contribution of manufacturing unit operations in the production of biologicals and biopharmaceuticals is recognized as a critical component in the triad of safety measures employed to effect prion clearance and contribute to product safety from a prion clearance standpoint. A model prion clearance study has yet to be designed; in it's absence we make use of a best fit approach taking into consideration the state of current knowledge and the uniqueness of the manufacturing process. Decisions to be made when designing a process validation study include the kind of spike to be use (hamster- or mouse-adapted sheep scrapie strain, murine-passaged bovine agent), high versus low concentration of spike, the type of spike used (crude, microsomal, detergent-solubilized, other) and the detection method employed (infectivity assay versus molecular assays). Regulatory guidelines advise that the clearance potential of a manufacturing process must be evaluated in light of available data. To-date, prion clearance studies for licensed manufactured products are not mandated unless specific claims are made.

CONCLUSIONS

Clinical acceptability of any biological or biopharmaceutical is concomitant with risk assessment and guided by risk benefit analysis. The unique considerations associated with virus/prion safety issues necessitate a pragmatic paradigm and a "case-by-case" approach. Causality assessment and management approaches for drugs cannot be directly applied to biologicals. For a conventional drug, benefit-risk assessment usually entails investigating the problem and collating a body of data to determine conclusive evidence beyond reasonable doubt for attributing the adverse event to the drug. For biopharmaceuticals,

every single reported case of suspected virus transmission must be considered as a potential indicator of an infectious batch, with the inherent risk of transmitting the disease to hundreds or thousands of patients (Additionally, suspected iatrogenic transmission of viruses (HIV, HAV, HBV, HCV) requires causality assessment because viruses can also be further transmitted through other exposures (e.g., sexual transmission).

Technological advances, demographic and societal changes, and subtle changes to our ecosystems will continue to leave us vulnerable to new or re-emerging viruses, as, for example, West Nile Virus. While even a minimal risk is viewed as unacceptable in the case of transfusion-transmitted viral infections and TSEs, the ideal zero risk goal must be balanced with the consequences of severe shortfalls of treatments which may be life saving. In the final analysis, while application of different numeric constructs have their place in estimating and ranking risk, protecting patients is paramount and should be the ultimate deciding factor in any risk management paradigm.

The TSE crisis and the sporadic reports of new or reemerging viral threats epitomize the difficulties in risk management when the nature of the risk and absolute risk levels are unknown. Our only recourse is a constant vigilance combined with pragmatic regulations and guidelines that take cognizance of the latest scientific and technical information.

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21

A Rapid Method for Purifying *Escherichia coli* β-Galactosidase Using Gel-Filtration Chromatography

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INTRODUCTION

This chapter is based on practical experiences within the Bio-processing course held at the BioNetwork Capstone Learning Center. The primary purpose of the Bio-processing course is to familiarize students with the major unit processes that comprise the upstream and downstream elements of a typical industrial bio-manufacturing operation, from both a theoretical and practical perspective. The experimental component of this course was designed to provide realistic, hands-on experience with these unit processes. An advantage of this course is that much of the equipment and instrumentation used is smaller in scale and generally more approachable than their larger and generally more complicated industrial counterparts.

Starting with 2.4 L of an overnight culture of a genetically engineered *Escherichia coli* strain students can visualize a 99.5% homogeneous preparation of the target protein, β-galactosidase, in a sodium dodecyl sulfate (SDS)-polyacrylamide gel within a 5-day period. The specific unit processes discussed and demonstrated include: bioreactor operation and control, target protein detection and monitoring, cell harvesting, cell-disruption, protein concentration and dialysis, protein separation by gel-filtration chromatography, and protein characterization using SDS-polyacrylamide gel electrophoresis.

Figure 1 is a flow-diagram of the overall experimental sequence for the course. As indicated in Figure 1, a bench-top bioreactor/fermentor may be replaced by several one-liter Erlenmeyer flasks. As a consequence, laboratories with limited equipment budgets can negotiate this course without compromising the quality of the final results.

THE TARGET PROTEIN: β-GALACTOSIDASE

Historical Perspective

The protein chosen as the target is β-galactosidase (EC 3.2.1.23) from the bacterium *E. coli*. Beta-galactosidase (lactase) hydrolyzes lactose and other β-galactosides into their constituent monosaccharides (Wallenfels and Weil, 1972). It is widespread in nature,

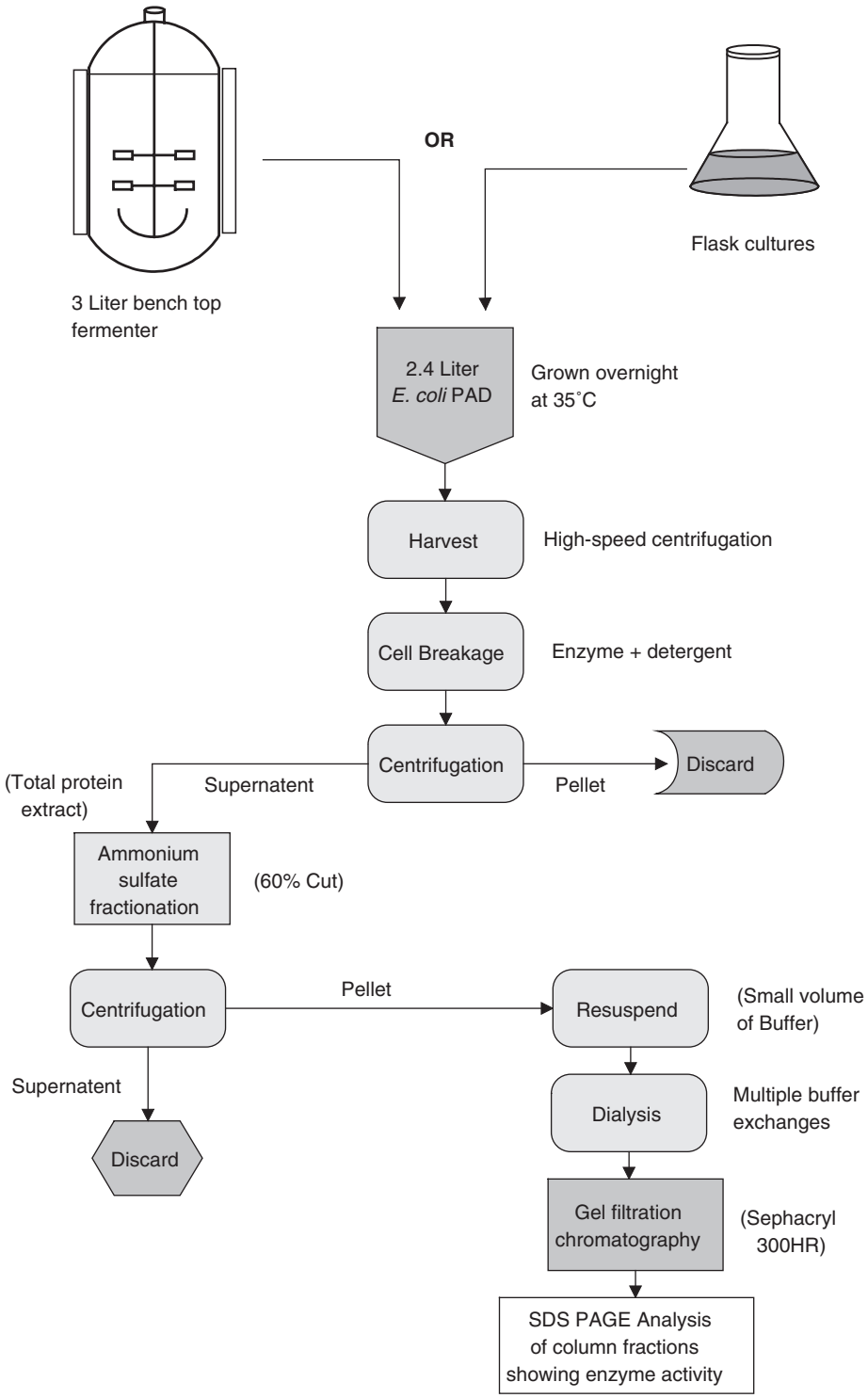


FIGURE 1 Flow chart for bio-processing course.

found in microorganisms, animals and plants. This enzyme is one of the gene products of the *lac* operon and, as such, has a unique place in the history of molecular biology. It is an inducible enzyme; *E. coli* synthesizes β-galactosidase only when its inducer, lactose, is present. The molecular mechanism for this adaptive phenomenon posed a thorny and intriguing problem that attracted the interest of two French scientists, Jacques Monod and Francois Jacob, working at the Institut Pasteur in Paris in the late 1940s. Relying on time-honored, classical techniques of genetically analyzing carefully selected mutants (molecular biology had not yet been invented), Jacob and Monod postulated (*i*) that a specific repressor molecule exists that binds near the beginning of the β-galactosidase gene at a specific site called the operator and that, by binding to the operator site on the DNA, it sterically prevents RNA polymerase from commencing the synthesis of β-gal messenger RNA, and (*ii*) that lactose acts as an inducer which, by binding to the repressor molecule, prevents the repressor from binding to the operator. Thus, in the presence of lactose, the repressor protein is inactivated and the messenger RNA is made. Upon removal of lactose, the repressor regains its ability to bind to the operator DNA sequence thereby switching "off" the *lac* operon and the synthesis of β-galactosidase.

This elegant hypothesis, explaining the adaptive phenomenon in *E. coli,* became known as the operon model of gene regulation and earned Francois Jacob and Jacques Monod the Nobel Prize in Physiology or Medicine in 1965 (Pardee et al., 1959; Jacob and Monod, 1961).

Molecular Characteristics

The functional form of β-galactosidase is a tetramer of four identical subunits, each consisting of 1023 amino acid residues (monomer molecular weight = 116,000 Da). Each monomer contains an active enzymatic site and they appear to be independently active. The tetramer (molecular weight = 465,412 Da) contains four catalytic sites that show no cooperativity or allosteric effectors (Juers et al., 2001).

The enzyme has three activities that ultimately result in the complete breakdown of the disaccharide lactose into galactose plus glucose. First, β-galactosidase cleaves lactose into galactose plus glucose. Second, the enzyme acts as a transglycosylase, converting lactose into allolactose. Third, it hydrolyzes allolactose into galactose plus glucose. Historically, it has been a puzzle as to why the β-galactosidase protein is so large and why it needs to be a tetramer. The recent elucidation of this enzyme's multiple and sequential activities may help explain its structural complexity and large mass (Juers et al., 2001).

Commercial Uses

In addition to its significance in the history of molecular biology, and the crucial role played in unraveling the nuances of gene regulation, β-galactosidase (lactase) has both medical and commercial applications (Waites et al., 2001). Lactase may be used as a reagent for determining lactose levels in blood and other biological fluids. In addition, β-galactosidase has important applications in food processing. Of special interest is its use in the treatment of milk to meet the needs of a large percentage of the world's population afflicted with lactose intolerance. The enzyme has been formulated in a pill form; one such product is sold under the trade name Lactaid.™ It is also used in ice cream manufacture, preventing the "sandy" texture caused by lactose crystals.

Why β-Galactosidase

This particular protein has a host of advantages to recommend it as the target product for our course including, (*i*) it is a very stable and well-characterized protein, (*ii*) its enzymatic activity can be easily and accurately assayed, (*iii*) it is unusually large and therefore amenable to purification in a single gel-filtration chromatography step, (*iv*) it is a therapeutically useful protein that is used to treat people who suffer from lactose intolerance, and (*v*) it is widely used in the food processing industry.

Assay for β-Galactosidase Activity

One advantage for choosing this particular enzyme as the target protein for this course is the fact that a well-established assay exists for β-galactosidase activity (Laderberg, 1950). A task that an entry-level bioprocess technician might be expected to carry-out is to monitor a fermentor or bioreactor for product yield and chances are good that this task will involve an enzymatic assay. Throughout this course, students gain valuable practical experience performing enzyme assays as well as interpreting and plotting their results.

A simple assay for β-galactosidase was originally devised by Laderberg, (1950) and later formalized by Miller, (1992). In order to assay enzyme levels, it is first necessary to permeabilize bacterial cell walls and membranes because β-galactosidase is an intracellular enzyme.

Briefly, small aliquots (0.1–5 ml) of an overnight culture are added to enzyme assay buffer (final volume $= 1.0$ ml) and exposed to chloroform and SDS. The cells are vigorously agitated followed by the addition of a 4% solution of orthonitrophenyl-β-D-galactoside (ONPG) and a second agitation step. Tubes are incubated at room temperature. ONPG is a chromogenic substrate of β-galactosidase that, when cleaved, produces an intense yellow color (Ausubel et al., 1992). The greater the enzyme concentration the quicker the yellow color appears and the more intensely yellow the solution ultimately becomes over time. The reaction can be stopped by the addition of a 1.0 M sodium carbonate solution which raises the pH to the point where the enzyme is no longer active.

The Quantitation of β-Galactosidase Activity

The intensity of the yellow color is quantified by determining its absorbance at a wave length of 420 nm in a spectrophotometer. The original cell density (OD_{600}), the volume of cells assayed, the absorbance value at 420 nm and the incubation time—post-ONPG addition—can be combined to determine the units of β-galactosidase activity according to the following formula (Miller, 1992; Becker et al., 1996):

$$\frac{1000 \times \mathrm{OD}_{420}}{\mathrm{t} \times \mathrm{V} \times \mathrm{OD}_{600}} = \text{units of } \beta\text{-Galactosidase activity}$$

where $t =$ the time of ONPG reaction (in minutes),

$V =$ volume of culture (in milliliters),

$OD_{600} =$ cell density immediately prior to the assay.

Despite the molecular elegance that *E. coli* manifests in its control and regulation of β-galactosidase synthesis, it normally produces very modest amounts of this enzyme.

So little, in fact, that Anfinsen and his colleagues needed to propagate *E. coli* in 300-L lots in order to accumulate sufficient protein for subsequent analytical studies (Craven et al., 1965). The advent of recombinant DNA technology and gene cloning

techniques has provided the means for scientists to construct custom-made bacterial strains possessing whatever trait or traits a researcher might find useful; *E. coli*-PAD is a prime example of the usefulness of recombinant DNA technology.

This project would not have been possible without two critical tools namely, (*i*) access to a genetically modified strain of *E. coli* and (*ii*) the technique of gel-filtration chromatography. First we will consider the genetically manipulated bacterial strain.

THE PRODUCTION STRAIN: *E. COLI*-PAD

Strain Construction

E. coli-PAD (MC4100 /*ppiA'-lacZ*$^+$) harbors a recombinant plasmid, pRS415, especially constructed to over-express the enzyme β-galactosidase. In this strain, *lacZ* (the structural gene for β-galactosidase) is under the transcriptional control of the *ppiA* promoter, a promoter that is highly expressed in Luria–Bertani (LB) growth medium. Details of the construction of the *ppi'-lacZ* fusion, using plasmid pRS415, is beyond the scope of this discussion however, the experimental details can be found in DiGiuseppe and Silhavy (2003) and Simons et al. (1987).

Strain Maintenance

E. coli-PAD is a thiamine auxotroph (Dr. Natividad Ruiz, personal communication) and must be grown in LB broth or agar. It will not thrive on general-purpose growth media such as trypticase soy broth or nutrient broth. In addition, this strain must be grown in the presence of ampicillin at a concentration of 125 μg/ml. The antibiotic is necessary to provide positive selective pressure for plasmid pRS415. Furthermore, it is a good idea to propagate this strain on LB agar containing 20 μg/ml x-gal. X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactoside) is a chromogenic substrate of β-galactosidase that, when cleaved, produces a blue color (Ausubel et al., 1992). X-gal is commonly used for detecting β-galactosidase made by recombinant vectors such as plasmid pRS415. Individual bacterial colonies that over-produce enzyme are varying shades of blue and the bluest of these colonies were preferentially selected for sub-culturing and subsequent experiments.

Strain Performance

In order to determine the extent to which *E. coli*-PAD over-expresses β-galactosidase, equivalent numbers of permeabilized cells from an overnight culture of *E. coli*-PAD and a wild-type *E. coli* strain were assayed for enzyme activity. Because β-galactosidase is an inducible enzyme, its synthesis needed to be induced in the wild-type strain thus, IPTG (isopropyl-l-thio-β-D-galactoside) was added to the culture at a final concentration of 1.0 mM. IPTG, unlike lactose, is non-metabolizable and acts directly by inhibiting the *lac* repressor. It is the generally preferred inducer for molecular studies involving the regulation and control of the *lac* operon. By contrast, *E. coli*-PAD requires no induction because the production of β-galactosidase, driven by plasmid pRS415, is constituitive.

The results of three independent assays showed that the genetically engineered strain, *E. coli*-PAD, synthesized approximately 45-times more β-galactosidase than did its wild-type counterpart. By extrapolation, a 2.4-L production culture of *E. coli*-PAD would be roughly equivalent to 108 liters of an induced, wild-type *E. coli* production

culture. This impressive level of enzyme over-production played a crucially important role in the successful purification of the target protein.

As mentioned earlier, the production of significant amounts of β-galactosidase would not have been possible without the over-expressing bacterial strain and the technique of gel-filtration chromatography. We will now discuss the chromatography component of the purification strategy.

GEL-FILTRATION CHROMATOGRAPHY

Introduction

Among the chromatographic techniques commonly used for protein purification, gel-filtration chromatography (also called gel-permeation, molecular sieve, gel-exclusion, and size-exclusion chromatography) is unique in that fractionation is based on the relative size of the protein molecule. In contrast to other methods such as ion-exchange, affinity, or hydrophobic interaction chromatography, none of the proteins or polypeptides is retained by a gel-filtration column. Since no binding is required and harsh elution conditions can be avoided, gel-filtration chromatography rarely inactivates enzymes, and is frequently used as the first step in a protein purification scheme. The disadvantage of non-binding however, is the fact that this property may limit the resolution of this type of chromatography. For example, Stellwagen (1990) has estimated that fewer than 10 proteins can be resolved from one another in the eluent from any gel-filtration column.

Principles of the Technique

Gel-filtration chromatography is a method for separating proteins and polypeptides based on their size. The chromatographic matrix consists of porous beads, and the size of the bead pores defines the size of the macromolecules that may be fractionated. Beads of differing pore sizes are available, allowing proteins of different sizes or, more precisely, hydrodynamic diameters, to be effectively separated. For the sake of this discussion, hydrodynamic diameter refers to the diameter of the spherical volume created by a protein as it rapidly tumbles downward in a buffer solution (Stellwagen 1990).

To start the process, a mixture of proteins or polypeptides is applied in a discrete volume or zone at the top of a gel-filtration column and allowed to percolate through the column matrix. Those proteins or polypeptides that are too large to enter the bead pores are excluded, and elute from the column first (i.e., the green circles shown in Fig. 2). A useful way to grasp the principle of this separation technique is to think in terms of buffer accessibility. Since large molecules do not enter the beads they have a smaller volume of buffer accessible to them namely, only the buffer surrounding the beads. As a consequence, large macromolecules move through the column faster and emerge from the column first. Proteins whose hydrodynamic diameter is small relative to the average pore diameter of the beads will access all of the internal volume and are described as being included in the gel matrix and emerge from the column last (i.e., the orange symbols depicted in Fig. 2). Proteins or polypeptides whose hydrodynamic diameter is comparable to the average pore diameters of the beads will access some but not all of the internal volume and are described as being fractionally excluded (i.e., the blue triangles shown in Fig. 2). These macromolecules elute from our rather idealized column in a middle position with respect to the other two proteins.

A diagrammatic representation of the principle of gel-filtration chromatography is shown in Figure 3. Molecules of different sizes in the far left column are separated

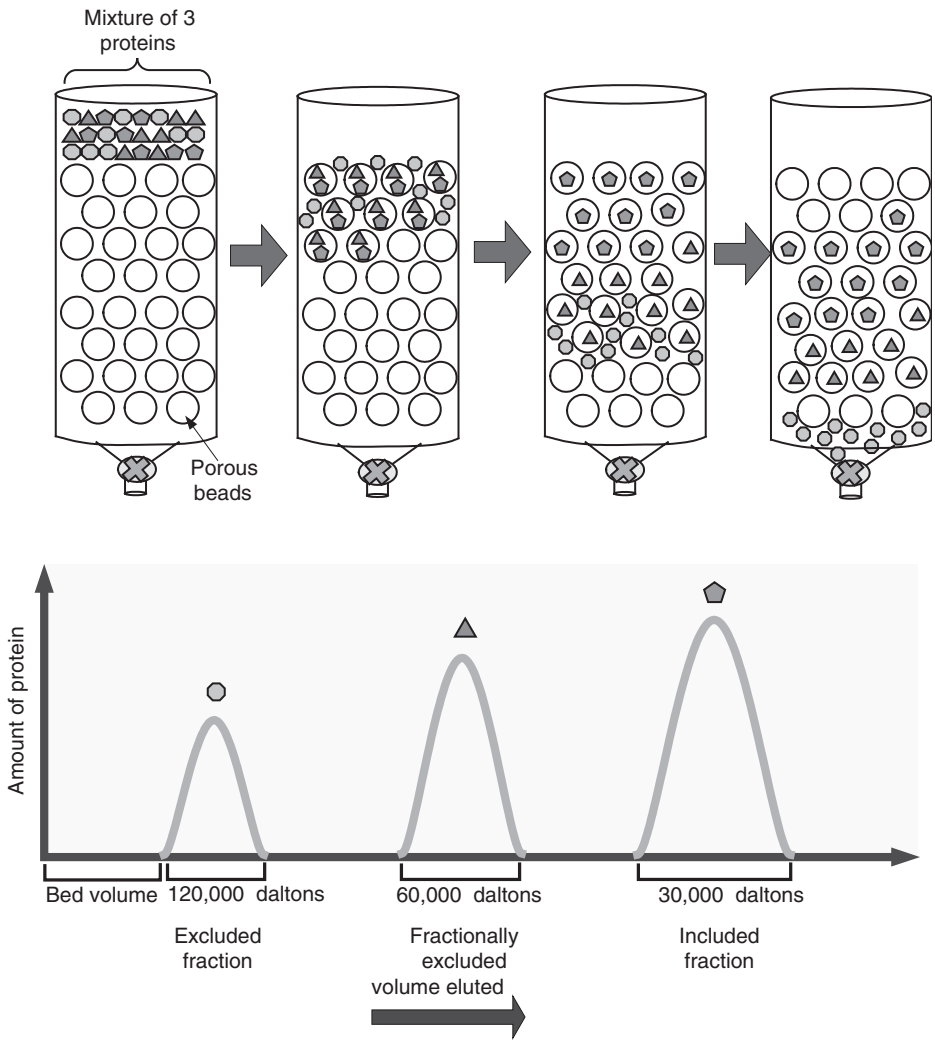


FIGURE 2 Principle of gel-filtration chromatography.

according to size during migration through the gel-filtration matrix depicted in the three columns to the right.

Table 1 provides a list of some of the commercially available gel-filtration matrices and their respective chemical compositions.

Equipment Needed for Gel-Filtration Chromatography

Figure 3 is a depiction of the equipment required for gel-filtration chromatography. The requirements are relatively modest, but laboratories with robust equipment budgets may opt for more sophisticated systems. For example, G.E. Healthcare sells a compact chromatography system called AKTA™ which is designed for one-step purification of proteins at a laboratory scale. This unit provided significant advantages in terms of speed

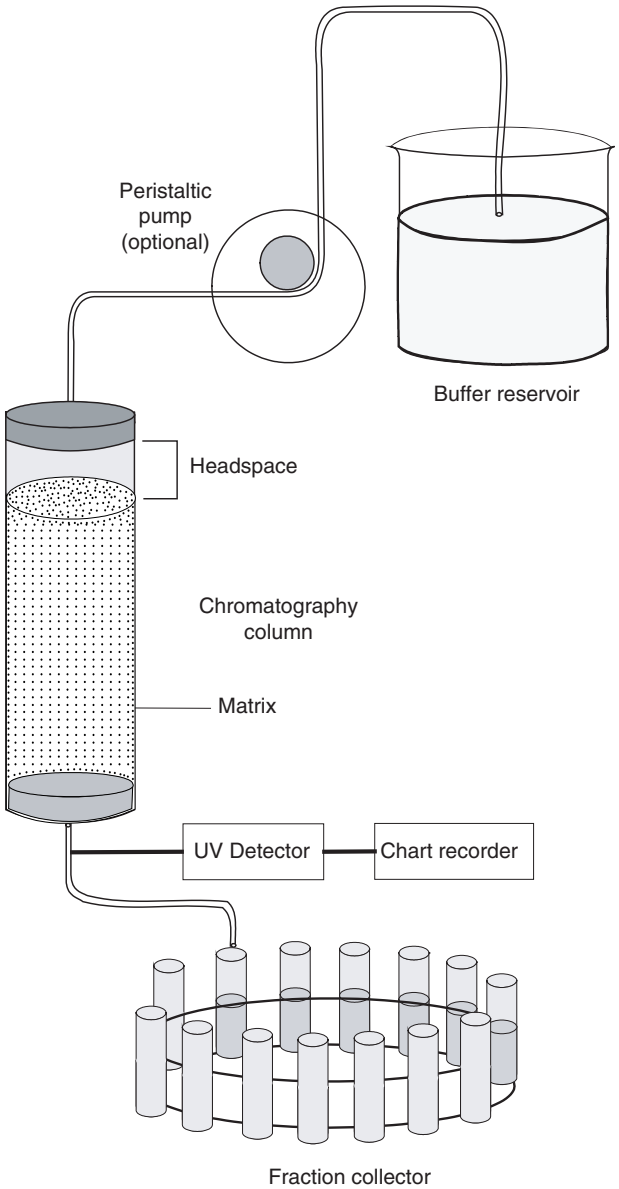


FIGURE 3 Equipment used in column chromatography.

and sample resolution. The subsequent results shown and discussed in this document were derived from the AKTA™ system. By contrast, earlier, test-of- concept experiments were done using a 30 year-old LKB Uvicord S UV monitor and an equally ancient LKB RediRac fraction collector and these instruments performed almost as well as the more automated and technically sophisticated chromatography system.

The heart of a gel-filtration chromatographic set-up is the column, which generally consists of a glass cylinder and a column support. Columns for gel-filtration are generally long and narrow, but the diameter should be at least 1.0 cm, so that potential anomalous effects from the protein and buffer interactions with the column wall can be avoided. The

TABLE 1 Matrices Used in Gel-Filtration Chromatography

Trade name	Supplier	Chemistry
BioGel A	Bio-Rad	Cross-linked agarose
BioGel P	Bio-Rad	Cross-linked polyacrylamide
Sepharose	Pharmacia	Cross-linked agarose
Sephacryl HR	Pharmacia	Composite: polyacrylamide / dextran
Sephadex G	Pharmacia	Composite: polyacrylamide / dextran
Ultrogel AcA	IBF	Composite: agarose / polyacrylamide
Ultrogel A	IBF	Cross-linked agarose
Macsrosorb KA	—	Composite of porous kieselguhr and agarose

Source: From Ratledge and Kristiansen (2001).

column used in this experiment was 2.5 cm in diameter and 30 cm long. Normally, a chromatography column is equipped with a tight-fitting adaptor on the top to allow homogeneous and efficient delivery of sample and buffer matrix bed. A buffer reservoir in combination with a peristaltic pump is frequently employed in order to control the column flow rate with a high degree of precision.

An ultraviolet (UV) wavelength detector is generally included to monitor the absorbance of the eluting sample. The signal from the monitor can be sent to either a chart recorder or a personal computer for analysis. Finally, the eluting samples are directed to a pre-programmed fraction collector that sequentially collects aliquots of eluent according to either time or volume (Fig. 3). All of this equipment can be purchased as individual components or as an integrated chromatography system such as the AKTA™ system previously discussed.

The Matrix

The column matrix for gel filtration must be chosen carefully to allow the best resolved separation of the protein of interest from contaminating proteins or polypeptides. The matrix should be chosen so that the target protein's molecular weight falls near the middle of the matrix fractionation range or so that contaminating components are well resolved from the macromolecule of interest. Table 2 outlines the fractionation ranges of a variety of commercially available gel-filtration matrices.

If a particular column matrix is not supplied as a pre-swollen slurry, the dry powder needs to be swollen in the appropriate buffer. Swelling is generally carried out by gently swirling the matrix in the appropriate buffer at room temperature. The use of a magnetic stirrer is discouraged because this strategy causes some of the beads to break into "fine" particles which subsequently cause irregularities in column packing or may reduce the column flow rate. Instead, slow agitation on a rotary shaker or occasional gentle swirling of the matrix using a clean glass rod is preferred. In addition, de-gassing of the matrix is strongly suggested in order to reduce the likelihood that air bubbles will form in the column.

The Potential Shortcomings of the Gel-Filtration Technique

The chief limitations of gel-filtration chromatography are that the separation may be slow and that the resolution of the emerging peaks is limited. The relatively low resolution occurs because none of the proteins or polypeptides is retained by the column during chromatography and because non-ideal flow occurs around the beads (Bollag, 1994). The speed of sample elution is limited primarily by the requirement for a long, narrow column

TABLE 2 Fractionation Range of Various Gel-Filtration Matrices

Trade name	Fractionation range[a] (kDa)
BioGel P-6	1–60
BioGel P-60	3–60
BioGel P-100	5–100
Sephacryl 100-HR	1–100
Sephacryl 200-HR	5–250
Sephacryl 300-HR	10–1500
Sephadex G-25	10–1500
Sephadex G-50	1.5–30
Sephadex G-100	4–150
Sephadex G-200	5–600
Sepharose CL-6B 1–6	10–4000

[a]The fractionation range defines the approximate protein and peptide molecular weights that can be separated with the matrix.
Source: From Bollag, (1994).

in order to permit sufficient component separation. This procedure may be accelerated by the use of matrices allowing faster flow rates and/or the use of pumps or high-pressure chromatography equipment, assuming the matrix in question is able to tolerate the additional pressure.

A further disadvantage involves the fact that size-exclusion columns are less forgiving compared with other types of chromatography. Gel-filtration columns must be poured and monitored with great care and attention to detail. For example, after packing a gel-filtration column, a visual inspection for air bubbles is necessary. Other things that can go wrong are outcomes such as matrix compaction and/or channeling. Compacting can generally be avoided by pouring the entire pre-equilibrated matrix slurry in a single step rather than incrementally. Vendors of chromatographic resins usually will provide helpful information to investigators regarding questions and problem-solving issues.

PRODUCTION OF β-GALACTOSIDASE

Cell Growth

Only 2.4 L of an overnight culture of *E. coli*-PAD were required for the successful negotiation of this experiment. As shown in Figure 1, one has the option of growing the production strain in either a small bench-top fermentor (New Brunswick Scientific BioFlo-110 Benchtop Fementor/Bioreactor System with a 3-liter capacity was used in this instances, others are Sartorius Biosystems BIOSTAT A Plus and Applikon BioBundle) or in individual Erlenmeyer flasks. Laboratories with limited equipment budgets may choose the latter option without comprising their results. In this case, individual 1 liter Erlenmeyer flasks were routinely used, each containing 600 ml of culture. In either case, cultures are grown overnight, with agitation, at 35 °C in LB broth supplemented with 125μg/ml ampicillin.

Cell Harvesting

After 18 h of growth, the culture was harvested by centrifugation. In this case, a Sorvall Super T-21 refrigerated super-speed centrifuge, an SL-250T fixed angle rotor and 250 ml

capacity centrifuge bottles. Cell pellets were washed with an appropriate isotonic buffer, containing magnesium ions (Steers et al., 1971) and the final cell suspension was re-centrifuged in a single pre-weighed bottle. The wet weight of the pellet was determined and the pellet stored at –20°C until further use. Generally, 2.4 L of an 18-h culture yielded 5–5.5 g of wet weight cells.

Cell Disruption

Cell disruption is a neglected area of bio-processing, as there has been relatively little significant innovation or progress over the last several decades (Waites et al., 2001). Cell disruption can be achieved both by mechanical (e.g., French press, high-speed ball mills, or sonication) and by non-mechanical means (e.g., simple treatment with alkali or detergents such as sodium dodecyl sulfate or Triton X-100).

Because β-galactosidase is an intracellular enzyme, the *E. coli* cell wall has to be disrupted in order to release the target protein. Ideally, one would prefer to use a non-abrasive cell-disruption method in order to minimize or avoid the destruction of the target protein. However, the breaching of the cell walls and outer envelopes of Gram-negative bacteria can pose thorny problems. In the first place, the liberation of large amounts of cellular DNA can increase the viscosity of the lysate to the point where it adversely affects the optimal flow through chromatography resins. As a consequence, a nucleic acid precipitation step or the addition of a nuclease during the cell-disruption process is usually recommended. Furthermore, target proteins released from cells are often subjected to degradation by cellular proteases and other hydrolytic enzymes. This adverse outcome can be minimized by the addition of enzyme inhibitors or by employing especially mutated host strains selected for reduced levels of proteases.

A commercially available protein extraction system called BugBuster™ distributed by Novagen Inc.(La Jolla, CA) to disrupt large numbers of *E. coli*-PAD cells has been used. This two-component cell-lysis system consists of a protein extraction reagent—a proprietary non-ionic detergent—plus a nuclease that Novagen calls Benzonase Nuclease.™ According to the manufacturer, this lysis cocktail is especially formulated to gently disrupt the cell wall of *E. coli*, resulting in the liberation of all soluble proteins with a minimum of protein denaturation.

Briefly, a 5.0-g (wet weight) cell pellet of *E. coli*-PAD was suspended in 25 ml of BugBuster™ extraction reagent followed immediately by the addition of Benzonase™ followed by the addition of phenylmethylsulfonyl fluoride, a potent protease inhibitor. The resultant cell suspension was incubated at room temperature for 20 min with gentle shaking. After incubation, the lysed cell suspension was centrifuged in order to remove cell debris. The resultant supernatant, referred to as the "total cell protein extract," was decanted into a clean tube and refrigerated.

Protein Concentration and Dialysis

A commonly employed first step in protein concentration is ammonium sulfate, $(NH_4)_2SO_4$, precipitation. This classic technique exploits the fact that the solubility of most proteins is lowered at high salt concentrations. As the salt concentration is increased, a point is reached where the protein comes out of solution and precipitates.

The "total cell protein extract" was adjusted to a final concentration of 60% ammonium sulfate by the addition of a super-saturated solution of this salt. Following a short period of stirring at room temperature, the cloudy solution (cloudy due to precipitated protein) was

refrigerated overnight. The resultant precipitate was pelleted by centrifugation and gently resuspended in a small volume of β-galactosidase assay buffer. This assay buffer contains a final concentration of 0.4 mM dithiethreitol (DTT). The reducing agent, DTT, is necessary to disrupt disulfide bonds, thereby disaggregating β-galactosidase into its constituent, enzymatically active monomers. The tetrameric form of this large enzyme (465,412 Da) would be excluded from conventional polyacrylamide gels. The suspension was exhaustively dialyzed against multiple exchanges of assay buffer and the resultant "mud"colored suspension was transferred to a clean tube or small bottle and refrigerated until further use.

ENZYME PURIFICATION USING GEL-FILTRATION CHROMATOGRAPHY

General Considerations

Chromatography is the technique of choice for high-resolution purification. These methods, normally involving glass columns containing chromatographic media, are universally used for the concentration and purification of protein preparations. In choosing the appropriate chromatographic technique a number of considerations must be taken into account. For protein products these factors include molecular mass, isoelectric point, hydrophobicity and inherent biological affinities. Each of these properties can be usefully exploited by specific chromatographic methods that may be scaled-up for use in an industrial unit-process context.

Choice of Matrix

As discussed earlier, one of the chief limitations of gel-filtration chromatography is that the separation may be slow and that the resolution of the emerging peaks is limited.

Resolution is limited because proteins do not bind to the matrix. As a consequence, the column matrix for gel-filtration must be chosen with great care to insure the best resolved separation of the target macromolecule from contaminants. The matrix should be chosen so that the molecular weight of the target protein falls near the middle of the matrix fractionation range. The fractionation range defines the approximate protein or peptide molecular weights that can be separated with that particular chromatography medium. Tables 1 and 2 provide information pertinent to the selection of column media.

Sephacryl-Hr Gel-Filtration Matrices

For this experiment Sephacryl HR (high resolution) medium has been chosen. As shown in Table 1, this particular gel filtration matrix is a cross-linked copolymer of allyl dextran and N, N-methylenebisacrylamide. According to Pharmacia, the narrow particle size distribution of this medium, together with its steep selectivity curve, make Sephacryl HR resolution matrices particularly useful for routine separations, especially when dealing with relatively large amounts of crude sample.

Sephacryl 300-HR was chosen for two specific reasons. First, the molecular weight of the β-galactosidase monomer (116,000 Da) fell within the fractionation range of this matrix (10–1500 kDa). The second reason was that Craven et al. (1965) had reported the efficient purification of *E. coli* β-galactosidase using a Sephadex G-200 column; the fractionation range for Sephadex G-200 is close to that of Sephacryl 300-HR (Table 2).

The large size of our target protein was advantageous with respect to its ultimate purification. Because of the relatively low resolution power of gel-filtration

chromatography, it is usually relegated to a late stage in the purification process after the total number of proteins has been narrowed down to a manageable few by ion-exchange and/or affinity chromatography. Gel-filtration chromatography, as a protein purification technique, is generally considered to be most effective when the desired protein target has a molecular weight either considerably larger or smaller than that of the majority of the proteins in a mixture. Because the 116,000 molecular weight β-galactosidase monomer is relatively large compared to the majority of other proteins synthesized by *E. coli*, we reasoned that the goal of purifying it in a single gel-filtration step was very likely.

Column Preparation and Sample Loading

A Kontes glass chromatography column ($30 \times 2.5\,cm^2$) was packed to a height of 28 cm with buffer-equilibrated Sephacryl 300-HR beads. The void volume (the volume of buffer external to the beads) of the column was determined by allowing a 0.2% blue dextran solution to percolate through the column and elute. The void volume of this column was calculated to be approximately 60 ml. This is a very useful value to determine because our exceptionally large target protein would be expected to filter near the front in a Sephacryl HR-300 gel-filtration column (Craven et al., 1965).

The packed column was secured into the AKTA™ chromatographic instrument. Approximately 3.0 ml of the dialyzed "total cell protein extract" was carefully layered onto the top of the column. The suspension was allowed to percolate into the beads, followed by the addition of 3–4 ml of assay buffer that was also allowed to percolate into the beads before the actual fractionation was initiated. The system was sealed and the fractionation begun. Buffer was introduced into the column throughout the run by way of the instrument's built-in peristaltic pump. The AKTA™ instrument was programmed to deliver a flow rate of 1.0 ml/min and to collect the column eluent in 4.0 ml aliquots. The column was allowed to run until approximately 2-times the void volume (around 120 ml had eluted. At the end of the run, the fraction tubes were collected and stored at refrigerator temperature until needed for further analysis.

ASSAYING COLUMN FRACTIONS FOR ENZYME ACTIVITY

The goal of this segment of the experiment was to identify the fraction tubes that contained pure β-galactosidase—free from contaminating proteins or polypeptides. To this end, the contents of selected column fraction tubes were assayed for enzyme activity employing the standard ONPG assay methodology previously described in detail.

Based upon the size of the β-galactosidase monomer (our target protein), it should elute close to the leading edge of the void volume. Given that the void volume was around 60 ml and that the eluent was collected in 4.0 ml fractions, we anticipated that we would detect β-galactosidase activity in the vicinity of fraction # 15, ($15 \times 4.0\,ml^2 = 60$ ml). Column fractions # 1 through # 28 were assayed for enzyme activity. It should be noted that in these particular enzyme assays, neither chloroform nor SDS was needed because we were dealing, in this case, with cell-free β-galactosidase—obviating any requirement to permeabilize bacterial cell walls. The 420-nm absorbance values were determined using a spectrophotometer, whereas the 280-nm absorbance values (the total protein profile) were measured and recorded automatically by the AKTA™ instrument.

The data generated by this analysis are shown in Figure 4. Clearly, the main peak of enzymatic activity emerged from the column shortly after the void volume front. Our prediction as to when the target protein would emerge was realized, in that

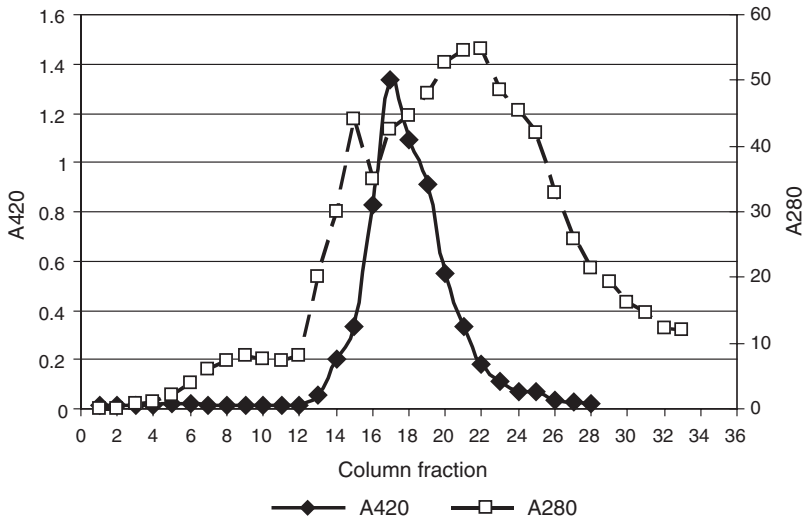


FIGURE 4 Gel-filtration on sephacry 300-HR of a 60% ammonium sulfate precipitated cell extract from *Escherichia coli*-PAD.

β-galactosidase activity (black squares) began to appear in column fraction # 14 and persisted to column fraction # 22. The bulk of the protein in the "total cell protein extract" (open squares), eluted from the column well after the enzyme activity peak. Interestingly, a large protein(s) appeared to elute before the β-galactosidase monomer. This apparent "retention" of the 116,000 Da β-galactosidase monomer in a gel-filtration column was similarly observed and reported, over fifty years ago, in Anfinsen's laboratory (Craven et al., 1965). These authors hypothesized that "this material may represent either a highly polymerized form of the protein or a fraction tightly bound to the nucleic acid components of the mixture." We made no attempt to investigate the nature of this phenomenon but, suffice it to say, the presence of this putative protein polymer or protein complex had no adverse effect on the purification of our target protein.

Fractionating the Enzyme Activity Peak

The experience gained from preliminary test-of-concept experiments showed that "pure" β-galactosidase monomer is confined to only one or two column fractions and those fractions resided exclusively within the leading edge of the enzyme activity peak. Based upon this knowledge, we sub-divided the enzyme activity peak into three component parts representing the leading edge of the activity peak; the *apex* of the activity peak; and the trailing edge of the activity peak. Referring to the profile shown in Figure 4, the leading edge segment would include column fraction # 15 through fraction # 17; the apex of the peak would consist of column fractions # 18 and # 19 and the trailing edge would include column fractions # 20 through # 22.

Concentration of Selected Column Fractions

Previous experience had also shown us that samples of eluent originating directly from the gel-filtration column contained an insufficient concentration of protein to make the

SDS-polyacrylamide gel electrophoresis analysis worthwhile. As a consequence, the pooled column fractions representing the leading edge, the apex and the trailing edge of the enzyme activity peak were concentrated.

This was accomplished by transferring the individual pooled column fractions into dialysis bags, sealing the bags and covering them—both top and bottom—with Ficoll-400™ powder in a glass dish. Ficoll-400™ is a non-ionic synthetic polymer of sucrose and is a registered trademark of Amersham Pharmacia Biotech.

The bags were incubated at room temperature and closely observed until the volumes had decreased by a pre-determined amount (generally 3- to 4-fold). Following volume reduction, protein values for each sample were determined using the Lowry technique. If the protein concentration of the samples was found to be in the range of 300–450 μg/mL, it could be safely assumed that sample loading volumes of 5–8 μL per lane would be sufficient to produce a satisfactory gel. Figure 5 is an example of such a gel.

PROTEIN ANALYSIS BY POLYACRYLAMIDE GEL ELECTROPHORESIS

The importance of the technique of SDS-polyacrylamide gel electrophoresis to monitor the step-wise progress throughout a protein purification scheme cannot be overestimated. Data derived from this important analytical tool directly informs an investigator whether he or she has purified their target protein to homogeneity—free from contaminating proteins and polypeptides.

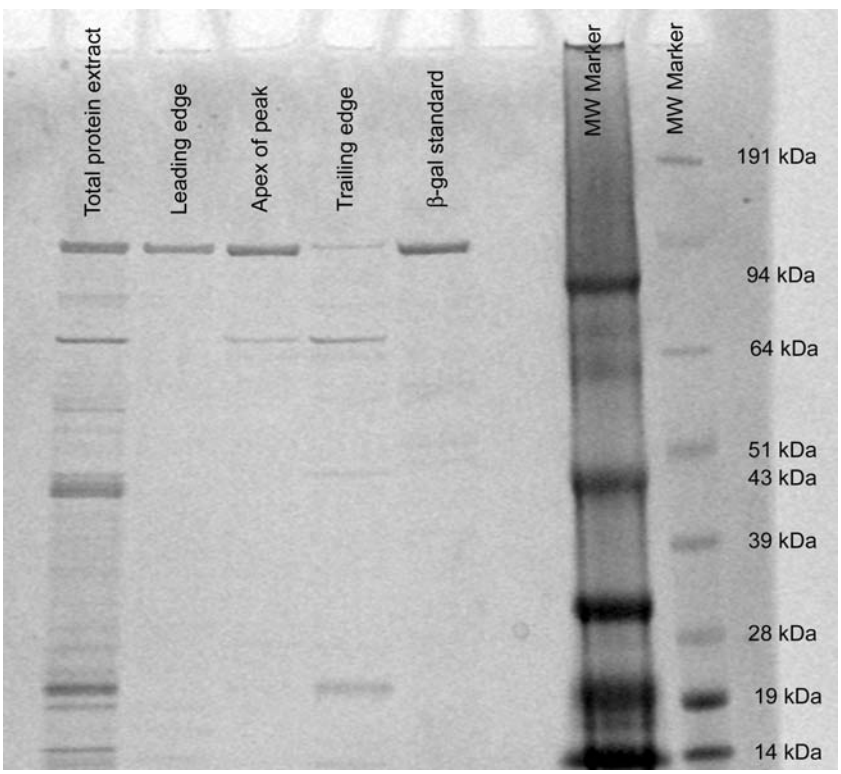


FIGURE 5 SDS-polyacrylamide gel analysis of selected sephacryl column fractions.

In the interest of full disclosure, it needs to be noted that the gel photograph shown in Figure 5 was not generated from the enzyme activity peak shown in Figure 4. Instead, it originated from a previous iteration of this course; one in which the gel-filtration patterns of β-galactosidase activity and total protein content were very similar to the profile seen in Figure 4. It is because of this "disconnect" that lanes in the SDS-polyacrylamide gel shown in Figure 5 were labeled: leading edge, apex of peak, and trailing edge rather than assigning specific lanes of the gel the corresponding column fraction number(s) as would normally be the case.

The gel electrophoresis system we chose was the NuPAGE Novex Bis-Tris Gel System™ supplied by Invitrogen Corporation (Grand Island, NY). This multi-component electrophoresis system comes with pre-mixed sample buffer, sample reducing agent, gel running buffer, pre-formed and ready-to-use 4–12% Bis-Tris Gels, as well as a compact electrophoresis apparatus the manufacturer has dubbed the XCell SureLock Mini-Cell.™

The following samples were loaded onto a Bis–Tris 4–12% gradient gel: a sample of the "total cell protein extract" (5.1 μg total protein); a sample of the leading edge segment of the enzyme activity peak (2.2 μg total protein); a sample of the apex of the enzyme activity peak (2.3 μg total protein); a sample of the trailing edge segment of the enzyme activity peak (1.9 μg total protein) and an authentic *E. coli* β-galactosidase standard (1.5 μg of total protein). The "total cell protein extract" sample was identical to the material originally loaded onto the chromatography column. It was included as a "baseline" indicator against which to judge the overall ability of the gel-filtration column to separate our target protein away from the host of contaminating proteins and polypeptides present in the original crude *E. coli* cell extract.

Samples were electrophoresed at 200 V until the tracking dye reached the bottom of the gel; this generally took 50–65 min. The gel was removed from the Mini-Cell apparatus and stained for approximately 30 min in a bath of Coomassie blue protein stain. The gel was de-stained overnight in a methanol/acetic acid/water solution with frequent exchanges of de-staining solution. Following de-staining, the gel was photographed and analyzed. Figure 5 is a photograph of a typical gel. An analysis of this gel revealed the following:

1. The lane containing the "total cell protein extract," derived from *E. coli*-PAD, contains 25–35 individual protein or polypeptide bands. The largest protein(s) visible in the "total cell protein extract" lane is slightly larger than 94 kDa according to the molecular weight protein markers.
2. The largest protein(s) synthesized by *E. coli*-PAD appears to co-migrate with the sample of authentic β-galactosidase monomer standard.
3. The leading edge segment of the enzyme activity peak contains pure β-galactosidase based on the observation that the only protein band visible in that lane co-migrates with authentic β-galactosidase monomer standard.
4. The apex of the enzyme activity peak contains a significant amount of β-galactosidase. In addition however, there also is a relatively minor amount of a contaminating protein or polypeptide with a molecular weight around 70 kDa.
5. By contrast, the trailing edge segment of the enzyme activity peak contains only a trace amount of β-galactosidase. Most of the protein in the concentrated trailing edge sample contains two protein species; one is the 70 kDa protein seen in the apex sample whereas the second contaminating protein species has a molecular weight of around 22 kDa.

In conclusion, SDS-polyacrylamide gel electrophoretic analysis of selected fractions eluting from a Sephadex 300-HR gel-filtration column revealed that "pure" β-galactosidase monomer resided exclusively in the leading edge of the enzyme activity

profile; 90–95% "pure" target protein could be found at the *apex* of the enzyme activity curve whereas the β-galactosidase monomer protein virtually disappeared in the *trailing edge* of the curve. This relatively "sharp" demarcation of protein species, of varying molecular weights, was a testimony as to how effectively our *single* gel-filtration column performed its role as a protein purification tool within the experimental conditions outlined in this document.

SUMMARY

Starting with 2.4 L of production culture, one has been able to purify the target protein, *E. coli* β-galactosidase, to 99.5% homogeneity in the course of four and one-half days. This was possible for two reasons: (I) the strain of *E. coli* we employed was genetically engineered to significantly over-express β-galactosidase compared to a normal wild-type isolate, and (II) the comparatively large size of our target protein made it amenable to purification using a single gel-filtration chromatography column.

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The experiments discussed in this chapter would not have been possible without the generosity and assistance of Dr. Steve Short and Leslie Walton—former colleagues of mine at the Burroughs Wellcome Co. (currently GlaxoSmithKline). Their gifts of incubators, UV monitor, fraction collector, and a variety of reagents and consumables were crucial elements in the developmental phase of this course. In addition, I am deeply indebted to Drs. Thomas Silhavy and Natividad Ruiz of the Department of Molecular Biology, Princeton University for allowing us to use their β-galactosidase over-producing strain of *E. coli*. Without their strain, this course would have remained an unrealized idea. I wish to acknowledge the support, both tangible and intangible, of my colleagues at the BioNetwork Capstone Center namely, Leonard Amico, Dr. Michael Morgan, Dr. Lin Wu and David Yarley. I greatly benefited from numerous discussions with Mike Morgan regarding the science involved in this purification scheme and the document itself greatly benefited from Len Amico's artful design skills which can be seen in Figures 1– 3. Finally , I am most grateful to Dr. Stephen C. Scott, President of Wake Technical Community College, who provided the laboratory in which the bulk of these experiments were carried out.

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22
Membrane Chromatography

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INTRODUCTION

New technologies in downstream process devices are enabling the bioprocessing industry to cope with new standards established by upstream manufacturing. About 10 years ago monoclonal antibody production titers were at 0.2–0.5 g/L. Current production titers are at 1 g/L and in the not too distant future these titers may reach 5–10 g/L (Wurm, 2004). These higher upstream yields will put pressure on downstream process unit operations, in particular chromatography columns. Columns used for large scale processing are already quite large. Greater product amounts will lead to even larger columns or batch cycling. Both of which are not appealing and for different reasons. Investment in larger columns and skids will have a negative impact on cost of goods. Cycling may prove to be a more economical solution with regard to capital and consumable expenditures. However, this will lead to increased validation efforts that will also have an economic imprint. Both of these solutions are cumbersome attempts to solve a real and impending problem. In recent years membrane chromatography has demonstrated it can offer a real solution to these forecasted bioprocessing bottlenecks. Current throughputs with membrane devices are dramatically greater than conventional columns on a gram per liter basis and are steadily improving. As will be discussed later, this is actually reducing the volume of sorbent required for certain chromatography unit operations. Thus, positively effecting overall process economy.

Development of large scale applications, small scale evaluations & scale up concepts and validation are aspects that have been covered in great detail in recent years. This is a product of the realization that membrane chromatography's capabilities can be adapted to provide a unique solution to current and future processing demands. Current large scale applications encompass capture and flow through steps, scale down designs have been optimized for more accurate scale up projections and validation studies show that this technology meets robust standards for biopharmaceutical processing.

Before pragmatic issues with membrane chromatography are discussed, it is imperative that basic principles and concepts of membrane chromatography and general chromatography details are covered in detail. Even though membrane chromatography has been available in the market for approximately 20 years, there are still large sectors that still do not realize what membrane chromatography is, how membrane chromatography devices operate and what precautions must be taken in order to ensure that membrane chromatography devices are evaluated properly.

Modern chromatography has been under development and use since the 1940s. Traditionally, this technology makes use of columns composed of innumerous bead like porous resins as a sorbent (the beads are approximately 100 μm in diameter). It is necessary to mention that the beads have ligands attached to their surface. Ligands are molecules that possess a region that has a specific charge or affinity. The bulk of the resins surface and inner pores is covered with ligands. This results in binding potential and will be discussed in detail below. The spherical beads are suspended in a solution that allows them to pour more easily into columns. The solution (often referred to as a slurry) is allowed to settle and is packed via hydrostatic pressure. Since the beads are semi-rigid, they will not fully compress under optimum pressure. This creates interstitial spaces between the beads. The entire mass of resin within a column is commonly called a resin bed, bed volume, or stationary phase. Columns have an inlet on the top and an outlet at the bottom. This allows for liquid or a mobile phase to pass through the interstices within the resin bed. The mobile phase is typically a complex mixture and concentration of molecules. Each species of molecule has a specific charge that can be bound by an oppositely charged ligand or moiety (region) that can be bound by affinity ligand.

Ion exchange chromatography (IEX) is characterized by using positively or negatively charged ligands to bind molecules within the mobile phase as they pass through the chromatography device. There are two general subsets within IEX. They are cation exchange chromatography (CEX) and anion exchange chromatography (AEX). These are named for the types of charged molecules they bind to under optimum conditions. Cation exchange chromatography utilizes resins with negatively charged ligands to bind positively charged molecules (cations). Anion exchange chromatography utilizes resins with positively charged ligands to bind negatively charged molecules (anions).

In either case, CEX or AEX devices are extremely useful as purification devices. Consider a mobile phase that is a complex mixture of thousands, tens of thousands or even millions of molecules. Only one species of molecules in the mobile phase is a product or target that must be purified from the others. Usually, the target molecule is well characterized and conditions in the mobile phase can be adjusted to allow the target to bind to the stationary phase a chromatography device. However, under the same conditions, contaminant molecules are allowed to flow through and are collected as waste. After loading the product, the chromatography device is washed. This flushes away any contaminants that remain within but not bound to the stationary phase. The wash is collected as waste. A series of salt or pH adjusted washes may ensue. This will strip of or elute any contaminants that are loosely associated with the ligands. However, these washes do not strip off the product. This action further removes contaminant molecules from the target product which remains on the stationary phase. At this point most or all of the contaminants that have a weaker attraction to the charged ligands have been removed. However, the product molecule and contaminants that have a stronger attraction to the ligands are still bound to the stationary phase. Finally, an elution condition is passed through the chromatography device. The elution has a well characterized salt/pH and it is optimized to strip off the target product but not the contaminants that remain on the stationary phase. This elution is collected as a separate fraction and contains highly purified target. The existing contaminants are removed by a high salt strip and collected as waste.

What was just described above was an example of positive capture. There is a mode of IEX that is complementary to positive capture and is rightly named, negative capture. This mode of chromatography is simpler to operate but just a powerful as its counterpart. During negative capture, target product flows through the chromatography device as

contaminants bind. Typically, this type of chromatography takes place when product is in high concentration; contaminants are in very low concentration and of only a few species. This allows for high polishing power near the end of a series of chromatography devices. This does not exclude the possibility of using negative capture with complex mobile phases. In fact negative capture is used for these applications too and is just as powerful.

Affinity chromatography is defined by using ligands that have a specific affinity for specific moieties of particular molecules. Immobilized Protein A is the most common use of affinity chromatography and is an excellent example how this mode of chromatography operates. Protein A is a ligand that has a strong affinity for the Fc region of antibodies. Under neutral conditions, the Fc regions of antibodies bind to Protein A. The Fc region will disassociate with Protein A when conditions in the mobile phase are changed to acidic (pH 2–3). This is a powerful purification tool when highly purified antibodies are desired and the mobile medium is a complex mixture of contaminant molecules and target antibody. Contaminants flow through and antibodies bind to Protein A, as the mobile phase passes through the chromatography device. Once the entirety of antibody is bound it can be stripped off or eluted as a separate fraction. The result is highly pure antibody.

PRINCIPLES AND BASIC CONCEPTS

Membrane chromatography operates under similar principles that define and characterize resin based chromatography. The major difference is that membrane chromatography has a stationary phase that is composed of a flat "filter like" membranes and resin based chromatography has a stationary phase that is composed of a spherical beads (Fig. 1). These beads are not solid. They have pores or crevices on the outer most surface. These openings on the outer surface lead to lumens within the spherical bead. About 90% of the beads available binding sites are within these lumens. The remainder of available binding sites (10%) are situated on the outermost surface of the bead. Chromatography resins are available in a variety of spherical diameters and pore sizes (also measured in diameter). The use of a specific resin diameter and pores size varies to suite the intended application. Both membrane and resin based chromatography platforms

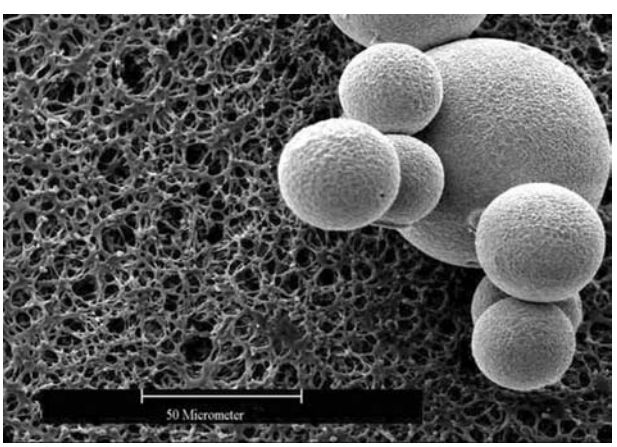


FIGURE 1 Photomicrograph with membrane chromatography stationary phase in the background and spherical resin's stationary phase in the foreground.

require ligands to bind either process impurities, target product or both. These ligands are attached to the outermost layer of the membrane if it is a membrane chromatography device or the ligands are attached to the outermost layer of a spherical resin and upon the outermost layer present within a resin's lumens. Membrane chromatography offers a variety of ligand types (Table 1). Within this platform, Ion exchange ligands are by far the most widely used. However, affinity ligands are also available for use with membrane chromatography devices.

Membrane chromatography is differentiated from conventional chromatography by its stationary phase. This difference dramatically affects how each platform is configured into a usable format. Membrane chromatography devices are available in disc, capsule or cartridge format. These devices are static with regard to bed volume and frontal surface area (FSA). However, there are a variety of small devices (1–10 ml) that can be used for small scale evaluations; mid sized devices (10–1000 ml) for pilot scale and larger devices (1000–5000 ml) for process scale (Fig. 2). Devices can operate in parallel or series to increase bed volume and overall binding capacity (Demmer and Nussbaummer, 1999). Final membrane configuration in cartridges and capsules can be in "layered spiral wound" or "pleated layer" format—which varies from vendor to vendor. The raw shape of both the spiral wound and pleated format is a hollow cylinder that can be referred to as a module. Modules are welded into a plastic housing in a fashion that is similar to normal flow filters. The top of the "layered spiral wound" or "pleated layer" format is sealed closed. The bottom is welded to a hollow disc or plate. The disc has a greater diameter than the "layered spiral wound" or "pleated layer" devices. This provides a bottom plate ledge. A dome-like housing is placed over the module and is welded to the bottom plate ledge. The above description constitutes a disposable capsule design. This design ceases at 30 inch elements. Larger devices have a central core that is located within the "layered spiral wound" or "pleated layer" device. This core occupies a void volume and is necessary for high resolution elution peaks, baseline separation and low elution volumes. In both instances, feed stream enters at the top of the housing and is typically driven by a peristaltic pump. This allows the mobile phase to pass through the module from the outside in. Once the mobile medium has passed through the membrane it occupies the void volume within

TABLE 1 Ligands Available in Membrane Chromatography Format

Ion exchange	
Anion exchange chemistries	
Quaternary ammonium (Q)	Strong basic
Diethylamine (D)	Weak basic
Polyethyleneimine (Mustang® E®)	
Cation exchange chemistries	
Sulfonic acid (S)	Strong acidic
Carboxylic acid (C)	Weak acidic
Affinity	
Protein A	
p-Aminobenzamidine pABA (pABA)	
Iminodiacetic acid (IDA)	
Cibacron blue	
Heparin	
Streptavidin	
Epoxy and aldehyde activated	



FIGURE 2 Pall XT 5000® (*left*) has 5 liters and Sartobind Mega is 1.62 liters of membrane volume. Sartobind Jumbo® (*not shown*) has 4.8 liters of membrane.

the cylinder. Continuous pressure from convective flow forces the mobile phase out through the opening in the bottom center of the housing. At this point the mobile medium has exited the capsule.

Not unlike conventional chromatography, membrane chromatography devices have a FSA, bed height and bed volume. These values can be attained from the supplier. Determination of these values is a matter of simple geometry and can be visualized in Figure 3.

As stated before, the raw shape of a membrane chromatography device is a hollow cylinder. Membrane volume (MV) is determined by first calculating the total volume of the entire cylinder (including the hollow core). Then subtract the volume of the inner core from the total volume.

$$\mathrm{MV} = V_{c1} - V_{c2} \tag{1}$$

where V_{c1} and $V_{c2} = \pi \times r^2 \times h$

V_{c1} is the volume of the entire cylinder,
V_{c2} is the volume of the inner core.

FSA is determined by the following equation:

$$\mathrm{FSA} = 2 \times \pi \times r \times h \tag{2}$$

Anatomy of a membrane chromatography device

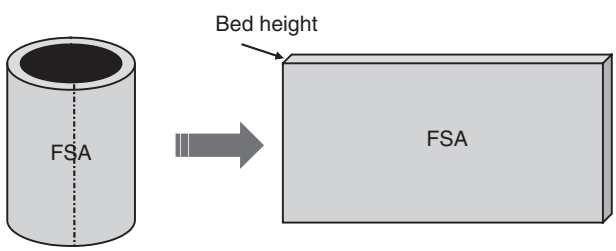


FIGURE 3 Cylinder on left represents raw shape of membrane. Frontal surface area (FSA) and bed height are seen when cylinder is cut and laid flat.

Inner surface area is less than FSA. This means the linear velocities are a fraction greater at the inner most portion of the membrane chromatography device. However, as will be discussed later, dynamic binding capacity is not affected.

Linear velocity, residence time and volume/min can be calculated if the aforementioned dimensions are known. The calculations are as follows:

Linear velocity:

$$\mathrm{cm}/h = [(n)(60\ \mathrm{min})]/\mathrm{FSA} \tag{3}$$

where n is nominal flow rate (ml/min) and FSA is frontal surface area.

Residence time:

$$t_r = [(60)(\mathrm{FSA})(h)]/n \tag{4}$$

where FSA is frontal surface area, n is nominal flow rate (ml/min), and h is membrane height.

Membrane volume/minute:

$$\mathrm{MV}/\mathrm{min} = n/\mathrm{MV} \tag{5}$$

where n is nominal flow rate (mL/min) and MV is membrane volume (ml).

The disparity in stationary phase design explains why membrane chromatography devices are well suited for high flow rate applications; purifying large molecules or dilute feed streams. Convective flow forces the mobile medium through the membranous stationary phase. At this point the ligands are 100% exposed to the molecules in the mobile medium as it passes through the stationary phase. These molecules have the chance to bind immediately to the ligands without diffusing into pores. Thus, molecules bind nearly instantaneously when they are exposed to the stationary phase. The same is true for elution conditions. When the mobile phase's pH or conductivity is adjusted, bound molecules disassociate from ligands. Convective flow forces the elution buffer through the membranous stationary phase. No diffusion is needed for the elution buffer to reach the bound molecules on the membrane's surface. Thus, molecules elute instantaneously when buffer conditions are changed.

Membrane chromatography design is optimal for purifying large molecules, viral vectors and vaccines. This is because the membranes have much wider pores than resins. The pore distribution of a membrane can be 0.2–5 μm (2,000–50,000 Å)—depending which supplier and format is used. A resin's pore distribution can range between 100–5000 Å or 0.01–0.5 μm. Even if a resin has a stated exclusion limit of 4×10^6 Da (5000 Å; 0.5 μm), dynamic binding capacity for large molecules can be dramatically less than binding capacity achieved with smaller molecules. As an example, thyroglobulin has a molecular weight of 660,000 Da. That roughly translates to 0.09 μm molecular diameter, well below the exclusion limit of 4×10^6 Da. However, the dynamic binding capacity (at 75 cm/h) for this molecule is only 2.5% of what it could be if using BSA, which is 67 kDa or 0.009 μm (Karlsson and Brewer, 1989). In general, pore sizes ranging from 30 to 50 nm (300–500 Å) are optimal for proteins with a molecular weight between 30,000 to 100,000 Da (Rounds et al., 1986). Larger proteins require pore sizes above 100 nm or 1000 Å (Regnier, 1984). In fact, only the outermost shell of the resin is utilized for binding capacity when large molecules are selected for purification. Some of the larger molecules may occupy the pores of a resin. However, these pores become occluded as large molecules diffuse into them. This restricts access to the available binding sites within the resin. As stated before, membrane chromatography provides pore sizes that range from 0.2 to 5 μm.

Even so, dynamic binding capacity (at 1200 cm/*h*) for thyroglobulin is only 83% of what it could be if using BSA. If the linear flow rate is 75 cm/*h*, dynamic binding capacity is 100% of what it could be if using BSA (Karlsson and Brewer, 1989). Molecular kinetics in the mobile phase best explains why dynamic binding capacity drops when binding large molecules with membrane chromatography devices. Greater mass results in greater inertia. Molecules with a greater mass will require a stronger opposing force to change their state of motion. As an example, a large molecule passes through the stationary phase in one device and a small molecule passes through the stationary phase of another device. Linear velocity is constant. If the ligand's and molecule's net charge remains constant, it will require more energy to change to state of motion of a large molecule towards a ligand than it would require for a small molecule (Fig. 4).

MEMBRANE CHROMATOGRAPHY DESIGNS

There are two major suppliers of membrane chromatography devices. Both provide similar devices but with minor differences observed in the stationary phase. These suppliers manufacture devices suitable for use in large scale manufacturing (Fig. 5). Table 2 and Table 3 show the variety of ligands, formats, volumes available for use and potential applications. The stationary phases are different, yet achieve the same end. One is a 0.8 μm polyethersulfone (PESU) membrane; other formats include 0.2 μm membranes. The membrane is stacked up to 16 layers in devices used for large scale processing and small scale devices. It is then pleated and put into capsule or cartridge format. The other has a stationary phase that is 3–5 μm nominal and is made of stabilized reinforced cellulose; other formats include 0.45 μm membranes. The membrane is spiral wound into 15, 30, or 60 layer capsules or modules. Both the PESU and cellulose base membranes are hydrophilic. This quality allows the membrane to be very low protein binding. Thus, any removal of proteins is by a chromatographic mode of operation. Of equal importance, this attribute ensures high product recoveries.

Layering of membrane sheets is important for proper function of the stationary phase within membrane chromatography devices. Multiple layers of membrane provides a torturous path for molecules in the mobile medium to pass through. This allows a minimal but necessary residence time for optimal dynamic binding capacity. At high

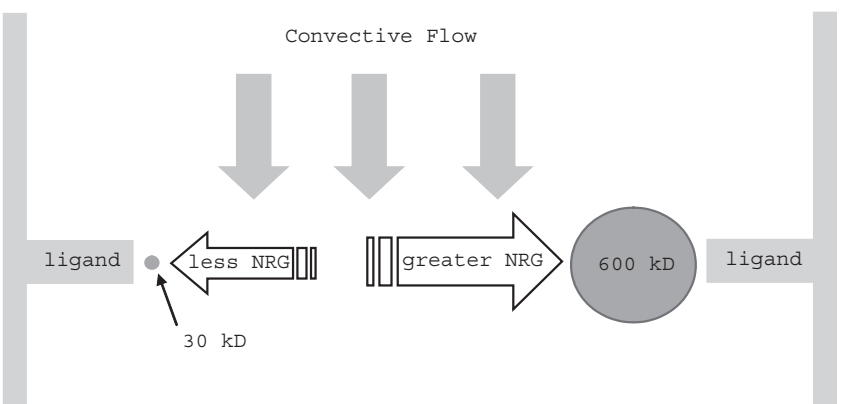


FIGURE 4 Larger molecules require more energy to change their state of motion.



FIGURE 5 10, 60 and 260 ml Mustang capsules (*left*) and 180, 360 and 540 ml Sartobind capsules (*right*).

linear velocities (> 1000 cm/*h*) a single membrane layer may not offer enough opportunities for molecules in the mobile medium to bind to the ligands on the microporous membrane. Consider a single pass as molecules in the mobile medium maneuver through a pore in a solitary sheet of membrane. The molecules that occupy the midpoint of the pore diameter are furthest away from the ligands. From this point the distance from the molecule to the ligands is quite significant and residence times can be as low as 1/10 of a second at 1000 cm/*h*. At that flow rate, the probability of a molecule occupying the center of the pore will bind to a ligand is low. Remember the ligands must provide sufficient opposing energy in order to change the molecules state of motion towards the ligand. The probability of binding increases if additional layers are added just beneath the first layer. More so, when the pores of the membrane layers are not aligned. When this occurs, a molecule in the mobile medium that may miss the opportunity to bind to ligands on the first layer, most likely will bind to a ligand on the next layers. This is because the convective flow that directs and carries the molecules in the mobile phase will move laterally in any direction in order to find the path of least resistance (Fig. 6). The probability that the molecule will bind to a ligand is directly proportional to the number of membrane layers. The concept increased binding capacity per unit surface area as directly proportional to number of layers was demonstrated by Knudsen et al. (2001). Binding capacity was measured as gram of antibody per liter of membrane. Results are summarized in Table 4 and clearly demonstrates that the relationship between dynamic binding capacity and layers of membrane.

POLISHING APPLICATIONS

Most biopharmaceutical production processes consist of several chromatography unit operations. Some schemes consist of up to five or more chromatography steps.

TABLE 2 A Summary of Membrane Chromatography Pore Size, Format, Size and Ligands Offered by Major Suppliers

Pore size μm	Number of layers/format	Volume (ml)	Ligands
Sartorius® Sartobind®			
3–5	1/disc	0.137	Q, S, C, D, (prt A, IDA, Ep, Ald, pABA)
3–5	3/disc	0.412	Q, S, C, D
3–5	15/cylinder in capsule	1	Q
3–5	15/disc	2.1	Q, S, C, D, (prt A, IDA, Ep, Ald, pABA)
3–5	5/disc	2.75	Q, S, C, D
3–5	15/cylinder in capsule	1, 7, 70, 180, 360, 540, 1620	Q & S
3–5	30/cylinder in capsule	4800	Q
3–5	15/cylinder in housing	35, 70, 140, 280, 560	Q, S, C, D, (prt A, IDA, Ep, Ald, pABA)
3–5	30/cylinder in housing	70, 140, 280, 560, 1120	Q, S, C, D, (prt A, IDA, Ep, Ald, pABA)
3–5	60/cylinder in housing	140, 280, 560, 1120, 2240	Q, S, C, D, (prt A, IDA, Ep, Ald, pABA)
(ligands in parentheticals are available with 0.45 m membrane)			
Pall® Mustang®			
0.2	3/disc	0.12	E
0.2	3/pleated in capsule	10, 40, 160, 320, 480	E
0.8	3/disc	0.18	Q & S
0.8	16/disc	0.36	Q & S
0.8	16/disc	5	Q
0.8	16/pleated in capsule or housing	10, 60, 260, 520, 780	Q & S
0.8	16/pleated in capsule or housing	5000	Q

Others may have as few as one or two. In either case, the mode of chromatography between the unit operations is typically complementary. A typical antibody process may consist of an affinity step followed by CEX/AEX, then hydrophobic interaction and size exclusion (Gottschalk, 2005). The complexity or number of chromatography steps is usually dictated by the nature of the target molecule and contaminants that are present in the mobile medium. The ultimate or later chromatography unit operations in a sequential scheme are traditionally committed to polishing applications (Fig. 7). More specifically, these unit operations are designed to remove any trace impurities that may remain in the process bulk, after it has been processed by earlier chromatography steps (Hanna et al., 1991). These impurities are divided into two categories: (*i*) product related impurities and (*ii*) process related impurities. Product related impurities are molecules that are associated with the final product but are not considered as such. Product dimers, fragments or clips, host cell DNA and proteins are considered product related contaminants or impurities. Leached ligands and media additives are considered process related contaminants or impurities. The bulk of these impurities express different

TABLE 3 List of Available Membrane Chromatography Ligands and Their Respective Applications.

Ligand	Mode of chromatography	Application
Quaternary ammonium	Strong anion exchange	Bind and elute (purification) polishing, negative capture. DNA & endotoxin removal, Virus clearance.
Diethyl amine	Weak anion exchange	Bind and elute (purification) polishing, negative capture. DNA & endotoxin removal, Virus clearance.
Sulfonic acid	Strong cation exchange	Bind and elute (purification) polishing, negative capture
Carboxylic acid	Weak cation exchange	Bind and elute (purification) polishing, negative capture
Protein A	Affinity	Bind and elute (purification) of antibodies
parabenzamidine (pABA)	Affinity	Bind serine based proteases
Imminodiacetic acid (IDA)	Metal chelate	Bind and elute of histine tagged proteins
E	polyethyleneimine	Endotoxin removal
Epoxy & Aldehyde	Na	Ligand immobilization

physiochemical properties than product. These disparities allow process scientists to separate contaminants from products.

Flow through Anion exchange (FT-AEX) chromatography is a powerful step that is commonly used for polishing applications. This mode of chromatography may also be referred as negative chromatography—not because of charge but because the product does not bind to the stationary phase (positive capture). During FT-AEX, negatively charged contaminants bind to positively charged ligands. All the while, product flows through the chromatography device. For this reason, product concentration does not change much after the intermediate bulk passes through the chromatography device. Throughput values for this type of chromatography step are defined at mass of product/L of sorbent (g/L). FT-AEX is convenient because most biopharmaceutical products

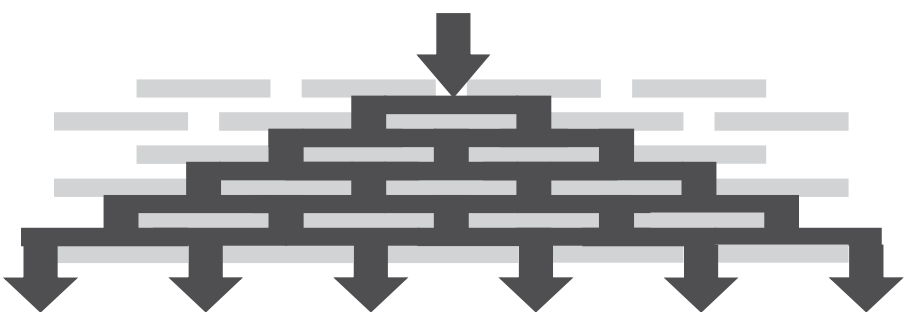


FIGURE 6 Cross sectional view of stacked membranes (*grey*). Flow dynamic of mobile medium (*black*) as it passes through multiple layers of membrane. Notice how the first layers are not saturated and final layers are.

TABLE 4 Relationship Between Number of Stacked Layers, Flow Rate ~4.5g/L at 450cm/hr, 4.5mS and pH 7.2, 0.5% Reo-3 Spike and Dynamic Binding Capacity

Number of layers	Bed height (mm)	Flow rate (cm/h)	~Dynamic binding capacity (g antibody/L membrane)
1	0.275	25	7
		125	
		250	
		500	
		750	
5	1.375	25	22
		125	
		250	
		500	
		750	
15	4.125	25	30
		125	
		250	
		500	
		750	

express a net neutral iso-electric point (pI) and most contaminants express an acidic pI (Fig. 8). The product will be positively charged if the pH of the mobile medium is below its pI. For neutral products the pH of the mobile medium can be as high at 6.5–7.0 and it will still be positively charged. However, contaminants with acidic pI's will be highly negative at neutral pH. Ligands of an AEX media carry a positive charge. Thus, a mobile phase that has a properly adjusted pH and conductivity will bind contaminants as product flows through. After this polishing step, the flow through is devoid of detectable contaminants or contaminants have been removed to a degree that they meet regulatory or in-house specifications. In addition to contaminant removal, FT-AEX chromatography can also be validated for virus clearance (Zhou et al., 2006).

It is known that dynamic binding capacity with membrane chromatography devices is less than bead based chromatography. However, polishing applications are not capacity driven because product flows through and only a few grams of contaminants per several thousand liters may be present for removal. For this reason, device size selection for this chromatography unit operation is based on flow rate and not capacity. Consider a process bulk intermediate of a monoclonal antibody that is at a volume of 3000L. The concentration of the antibody is 5 g/L and DNA/HCP are at 100 ppm or 500 µg/L (=15,000 g MAb and 1.5 g DNA/HCP in 3000L). Throughput capacities for *disposable* FT-AEX membrane chromatography are currently at >10 kg MAb/L of membrane (Mora et al., 2006). This is when there are very low levels of contaminants present in the feed stream. The 3000 L process intermediate will require 1.5 L of membrane for capacity of DNA and HCP. Volume is calculated by dividing total mass of product by throughput capacity. A 1.62 L membrane chromatography device contains the membrane volume that will accommodate this capacity. However, flow rate is still an issue. A 1.62-L device has a FSA of 4050 cm^2. Linear flow rates with FT-AEX membrane chromatography devices are typically around 500 cm/*h*. That equals ~33 LPM or 20 MV/min with a 1.62 L device. At that flow rate it will require 1.5 *h* to process 3000 L.

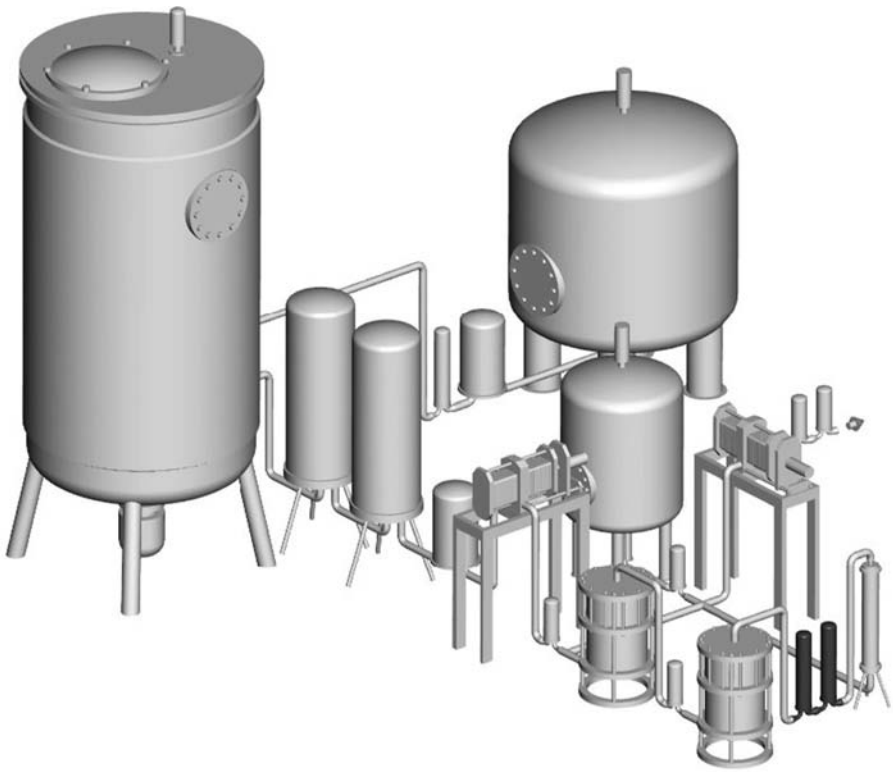


FIGURE 7 Typical biomanufacturing process. Polishing unit operations (*darkened*) are commonly towards the end of the purification process.

Of course conventional chromatography will have the same, if not better overall throughput when considering capacity for DNA and HCP. What would happen if a column was sized on impurity capacity alone? A column that is 1.5 L (10 cm diameter $\times$ 20 cm high) would take approximately $9 \times 24\,h$ days to operate at linear flow rates currently used in the industry (100–200 cm/*h*). Column diameters must increase in order to accommodate higher nominal flow rates—usually to keep unit operations within the time frame of one work day. As a result, chromatography columns for polishing are grossly oversized with regard to capacity. For instance, a 1.2 m diameter column (11,304 cm^2 FSA) will achieve a nominal flow rate of ~33 LPM at 175 cm/*h*. Flow through IEX columns have an average bed height of 20 cm creating a column that is > 200 L in sorbent volume. Roughly 200x larger than it needs to be for capacity alone.

The advantage of using a small volume, disposable membrane chromatography device can be visualized in overall process economy and ease of use. AEX resin columns and disposable membrane chromatography devices are both capable of trace contaminant removal and virus clearance. The difference between the two formats is load capacity at flow rates acceptable for large-scale manufacturing and disposability. Capacity and disposability are critical factors to consider when calculating unit operation costs for new products and processes.

Process lifetime can be up to 10 years: generally the lifetime of a product before generics may compete and erode its price. That 10-year process is assumed to encompass 400 batch production runs and column cycling up to 100 times (four columns, total).

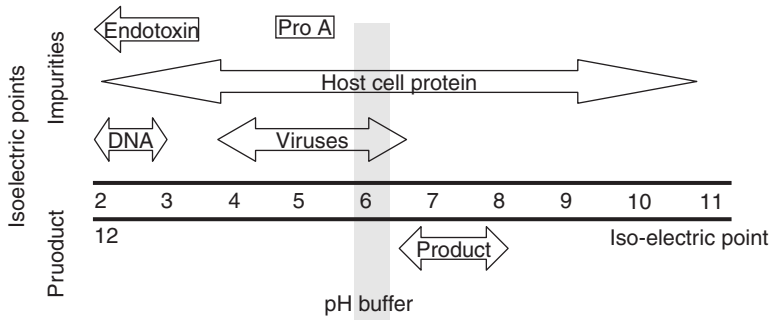


FIGURE 8 Approximate iso-electric points of product and impurities are listed above. If AEX chromatography is used, molecules below the pH will bind.

Consider that each batch production run stems from a 15,000-L bioreactor. MAb yield at cell harvest is 1 g/L CHO. Thus, each batch produces 15 kg MAb—assuming a 100% process yield. The column size will be 215–225 L, and the membrane chromatography device will be 1.6 L.

Cost models that compare disposable and column based chromatography have been developed and published in the recent past (Zhou and Tressel, 2006). Input data are from the aforementioned load capacity. Results demonstrate where cost benefits are and are not encountered. This economic model yields a 23% cost reduction when disposable membrane chromatography is used in place of resin-based chromatography. Overall operating costs for the FT AEX step are reduced in spite of increased consumable costs, although there is an economy of scale that was not considered in the evaluation. Media costs increase because a new membrane chromatography capsule is used for each chromatography cycle. Conversely, a column is reusable and can be cycled up to 100 times. BioPharm Services provides a cost model that allows end users to input their own data to a software program.

This program demonstrates that membrane chromatography costs break even to columns when membranes are loaded to 2 kg MAb/mL and 80% less than columns if loaded to 10 kg MAb/mL. The cost model considers all aspects of a Q FT AEX unit operation, including user interface, utilities, production, labor, consumables, materials, and capital equipment (Sinclair and Mongue, 2002). Dramatic reductions in buffer consumption, labor, and overhead are a direct result of smaller membrane chromatography devices. Column volumes—or in this case membrane volumes—are significantly reduced because a membrane chromatography device is 1.6 L, rather than 220 L. Smaller membrane volumes translate into less buffer volume required for equilibration and wash steps while eliminating cleaning steps. In fact, buffer consumption is reduced > 95%. Disposability eliminates significant upfront costs such as cleaning validation, a column lifetime study, assay development, packing studies, hardware, and columns. Those costs are not required with a disposable chromatography device. The cost benefits provided by reduced buffer consumption, processing time, and upfront costs overcompensate for the increased cost of membrane chromatography media cost as shown in Figure 9. Consideration of hold up volume is of equal importance and is directly related to the volume of the chromatography devices used for a unit operation. Large scale columns can be quite large with respect to volume. Larger columns require copious volume buffer flushes for optimal recovery or product. A smaller the chromatography device will require less buffer to flush residual product from the device. Thus, eliminating dilution of product or sacrificing product left behind on the chromatography device.

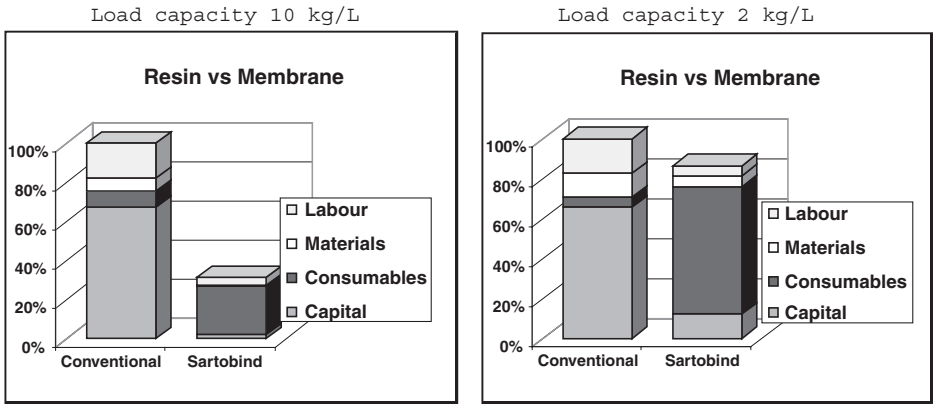


FIGURE 9 Graphic results from the Biopharm Services cost model. Component costs are added up to 100% percent for the column. Results show membrane chromatography is 80% more affordable than columns if load capacity is 10 kg MAb/L and that membrane chromatography breaks even if loaded to 2 kg MAb/L of membrane.

CAPTURE/PURIFICATION APPLICATIONS

In most instances the first chromatography step in a sequential chromatography scheme is designed to remove the bulk of process and non process related impurities. This chromatography step may also serve to concentrate the process stream into volumes that are more convenient to manage. When using IEX chromatography, pH and conductivity of the mobile phase is adjusted, so that product binds and impurities flow through. In order for membrane chromatography to be a viable option as a bind and elute device, products should be in low concentration; large in molecular weight (< 300kD) or both.

High flow rates enable membrane chromatography devices to concentrate dilute feed streams in a short period of time. This is critical when converting large upstream process volumes to smaller volumes that are easier to manage. Also, this strategy is useful to remove degrading agents or conditions from products in a short period of time.

5-L membrane chromatography devices demonstrate that 2000 L of filtered low titer harvest (~100 mg/L) can be processed at 10–12–MV/min or 50–60 LPM. Within one hour, product is bound, washed and eluted in concentrated form. Baseline separation between each of these fractions shows that resolution is good. Once again, resin based chromatography would require 1.2–2.0 meter diameter columns in order to achieve such flow rates. That would equate to a 200–300 L column to achieve the same task.

Purification of large molecules and particles also plays into the favor of membrane chromatography devices. Fibrinogen, plasmid DNA, viral vectors and vaccines all fall into the category of large biomolecules or particles. Some of these molecules may be just under the exclusion limit of chromatography resins. However, there is still the all too familiar case of occluding pores with only a few target molecules. Once, this occurs the internal surface area of the resin is useless for capacity.

SCALE UP PRINCIPLES AND REQUIREMENTS FOR LARGE SCALE PROCESSING

Large scale membrane chromatography devices have proven to be effective for trace contaminant removal (Walter, 1998). Conveniently, this unit operation can also be

validated for virus clearance. As discussed previously this application itself is described as a "flow through or negative capture unit operation" and throughput is defined by measuring the amount of product (mass) that can pass through a unit (volume or area) of membrane (g/L). This throughput value (g/L Q membrane) is determined by small scale experiments that accurately reflect large scale process conditions. In these experiments the feed material has a known amount of contaminants and product. DNA, host cell proteins (HCP or CHOP), leached protein A and endotoxins are the most common contaminants that are removed by Q membranes (strong basic anion exchanger). Contaminants are usually at the ppm (ng/mg of product) or ppb (pg/mg of product) level and product concentration can range from 5–10 g MAb/L.

The feed material is typically an antibody pool that is a mixture of contaminants and product. At this point in the process the contaminant concentration should be at trace levels (< 100 ppm). Contaminants bind to the Q ligands and product flows through as the feed material passes through the Q membrane capsule. Initially, the flow through should be devoid of contaminants. Eventually, the Q ligands will become saturated with contaminants. As this occurs, contaminants are detected in the flow through. Breakthrough has occurred when contaminants first appear in the flow through. Saturation or 100% breakthrough has occurred when the concentration of contaminants in the flow (C) through is equal to the concentration of contaminants in load (Co). It is up to the process developer to decide what levels of contaminants are acceptable in the flow through. These levels may be 0 or at the throughput amount before breakthrough. On the other hand, acceptable contaminant levels may be anywhere between breakthrough and 100% breakthrough. As a rule of thumb, HCP should be < 5ppm and DNA should be removed to 10 ng per dose in the final flow through pool—these are current accepted levels (Fahrner, 2001). These are the current specification given by regulatory bodies. Dose amounts range in quantity. Thus, acceptable levels of DNA in the flow through can only be claimed when the dose amount is determined. A high dose of MAb is 150 mg. That means, for every 150 mg MAb there can only be 10ng of DNA or 67 ppb.

SMALL SCALE EXPERIMENT SET UP

The goal of the small scale experiment is to accurately predict how much membrane (capacity) will be used and how long (flow rate) the application will take during large scale processing. Buffer conditions (pH and conductivity) suitable for the unit operation can also be determined during these experiments. The most effective conditions are neutral pH (0.5 to 1 unit below the pI of the product) and a conductivity of 5 mS/cm (20 mM NaCl).

The most representative scale down model is the best device to use for small scale experiments. This should have similar flow dynamics compared to process scale devices. This device will behaves similarly to large scale cylindrical devices and will allow for more accurate scale up. The smallest available evaluation scale device that has the same format as large scale devices is a nano capsule. Its total bed volume is 1 ml (36.4 cm^2) and it has a frontal surface are of 2.4 cm^2.

As discussed earlier, current throughputs for Q membranes are > 10.9 kg MAb/L of membrane. That translates to > 10.9 g of MAb/ mL of membrane–virus clearance values and trace contaminant removal are good at this throughput. Therefore, a 1 ml capsule can accommodate > 10.9 g of MAb. Average antibody pools that are loaded onto a

Q application are ~5 mg MAb/ml of buffer. A 1 ml capsule can process >2000 mL of antibody pool. The feedstock at this point in the process (post protein A and S column) is typically <98% pure.

Prefilter the load with a 0.45 or 0.2 μm membrane filter. This will ensure that the membrane will not foul. Take a sample of the load for contaminant detection assays.

The 1 mL capsule can be operated with a peristaltic pump at a flow rate of 20–25 ml/min (450 cm/*h*). Operating pressure for the experiment can range from 1–3 bar. If required a UV meter can be placed downstream of the 1 ml capsule. This meter will give a signal to a recording device that will measure UV 260/280. The meter will not be sensitive enough to discern between contaminants and product. However, it can detect product and is useful for initial recovery data.

The membrane must be flushed with at least 10 MV (10 ml) of equilibration buffer before use. This buffer should have a pH and conductivity that is similar to the load material (antibody pool). It is important that the 1 mL capsule is full of buffer after it has been flushed.

Load the antibody pool (~2000 mL) at 20–25 ml/min. Collect the flow through in 100 mL sequential fractions. These fractions are used for assay detection of CHOP, DNA, leached protein A and other contaminants.

Wash the membrane with at least 10 MV (10ml) buffer after the entire antibody pool has been processed. If a UV meter is in line, wash the membrane until the signal returns to baseline. Collect the wash as another fraction.

For a thorough experiment, strip the membrane with at least 10 MV (10ml) 2 M NaCl. Collect elution as a separate fraction. This will elute bound contaminants and can be used to confirm the levels of contaminants that were removed. The elution will also determine if any product was bound to the membrane during the experiment.

The fractions that were collected during the load, wash and elution will be assayed for contaminant removal and product recovery. Results from the load, wash and sequential flow through fractions should be at ppm or ppb level. The first fractions of the flow through should not have any contaminants. As the fraction volumes increase, there should be a point where contaminants are detected (breakthrough). Soon after breakthrough, the concentration of contaminants in the fractions will be equal to the load (100% breakthrough). Figure 10 demonstrates how plotting these data make up a breakthrough curve. Membrane capacity will be at a throughput volume or mass between breakthrough and 100% breakthrough. This depends on what contaminant level is acceptable.

It is imperative to differentiate between concentration of contaminants in the fractions versus the concentration of contaminants in the entire flow through pool. For instance: the final fraction concentration can be 50 ppm HCP in 100 ml or above maximum specifications. However, if all the fractions are pooled in 2000 ml, the concentration will be <5 ppm or below specification.

The above example for HCP and DNA removal can be used for scale up. The entire 2000 mL antibody pool (5 mg/ml) had and initial HCP & DNA concentration of 100ppm. This was loaded on a 1ml membrane with a FSA of 2.7 cm^2. The flow through pool had a HCP concentration of <5ppm and a DNA concentration <5 ppb. Thus, 10 g of MAb was able to pass through 1 mL of membrane and HCP/DNA was removed to below specifications. Flow rate was 20–25 ml/min or 450cm/*h*. This information is useful to calculate how much membrane will be used in large scale processing and how much time the unit operation will consume during large scale processing. Below are examples of how this is carried out.

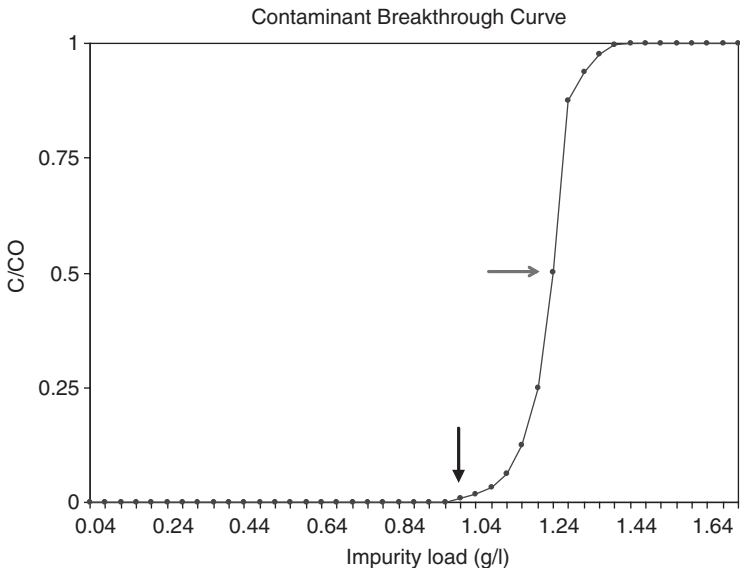


FIGURE 10 Breakthrough curve for CHOP. Black arrow indicates initial breakthrough, grey arrow indicates point at which flow though pool has 5 ppm impurities (at 50% BT).

CAPACITY

The ratio of product per L of membrane is known. Divide the total amount of product at large scale by membrane capacity to determine what size device can be used.

15 kg MAb/(10 kg Mab/L Membrane) = 1.5 L of membrane.

1.5 L of membrane can accommodate 15 kg of MAb.

PROCESSING TIME

The MAb pool is at a concentration of 5 mg/ml (= 5 g/L). 15 kg (= 15,000 g) total MAb yields a pool size of 3000 L. The FSA of a 1.62 L membrane is 4050 cm^2. The flux is 7.5 ml/min/ cm^2, multiply this value by the total FSA of the large scale device (4050 cm^2) to yield process flow rate. Divide total volume by process flow rate to determine process time. Add 30 min for preparation and buffer flushes.

Liner flow rate can also be used to determine process time. The linear flow rate for the small scale experiment was 450 cm/*h*. Use 4050 cm^2 as the divisor in the linear flow rate calculation and determine the process flow rate.

1. Flux method:
   (7.5 ml/min/cm^2) (4050 cm^2) = 30,375 ml/min or 30.375 LPM
2. Linear flow rate method:
   cm/*h* = [(ml/min)(60 min)]/surface area cm^2
   450 cm/*h* = [(**x** ml/min)(60 min)]/4050 cm^2
   x ml/min = [(450 cm/*h*) (4050 cm^2)]/60min
   x ml/min = 30,375 or 30.375 LPM

3. Processing time:
   3000 L/30.375lpm = 98.77 minutes
   98.77 min + 30 min for preparation and flushes = 128.77 min (2 h, 9 min)

1.5 L of Q membrane can process 15 kg of Mab. Initial HCP and DNA concentration will be 100 ppm. Final HCP & DNA concentrations will be < 5ppm and < 5ppb respectively. A 1.62 L capsule should be used to process 15 kg Mab. The MAb pool will be 3000 L. A 1.62-L capsule will have a flow rate of 33 LPM. The entire pool will be processed in 98.77 min. 30 min were added for preparation and buffer flushes. Thus, the total process time will be 128.77 min or 2 h and 9 min.

VALIDATION ASPECTS

Contaminant removal capabilities and single use concepts are the key drivers that characterize validation of membrane chromatography for GMP use. Single use eliminates the need to validate cycling. Proof of a consistent, defined level of contaminant removal eliminates the need for QC testing for contaminants (CPMP, 1997). This can happen only if the device that is removing contaminants demonstrates a power of removal that is below regulatory or in house specifications. If the performance is predictable and robust, there is no need to test for contaminants because the processing step has been validated for contaminant clearance below specifications. DNA and virus clearance are the two most accepted and validated applications for membrane chromatography devices thus far. 2001 was the first time a membrane chromatography device was used in a validated process for a market approved biopharmaceutical product (Galliher and Fowler, n.d.). The purpose for the validated polishing unit operation was DNA removal. However, the step was also validated for virus clearance. The virus clearance validation was scaled down to show effectiveness at 2,000 and 12,000 L production. Removal of DNA was validated at 3 LRV and virus clearance values for four model viruses was also achieved (Table 5). In 2004 the second membrane chromatography unit operation was validated for DNA removal and virus clearance (Martin, 2004). This too was for an approved product. Removal of DNA was validated at 5 LRV and virus clearance values for model viruses was also achieved (Table 6). Since 2001 numerous products have been validated for first in human and phase III clinical trails.

TABLE 5 Virus Clearance Values for 2,000 and 12,000 Liter Monoclonal Antibody Production Runs

Viruses	Size (nm)	Enveloped	Clearance by sartobind Q factor ($\log_{10}$) run 1	Clearance by sartobind Q factor ($\log_{10}$) run 2
SV-40: Simian virus –40	45	No	1.25 ± 0.46	1.34 ± 0.43
Reo-3: Reovirus type III	75–80	No	4.07 ± 0.50	3.62 ± 0.42
MuLV: Murine leukemia virus	80–110	Yes	3.80 ± 0.39	4.40 ± 0.56
PRV: Pseudorabies virus	150–250	Yes	3.97 ± 0.44	3.88 ± 0.38

TABLE 6 Virus Clearance Values for Large Scale Processing Runs

Virus	Size (nm)	Enveloped	Removal of virus from flow-through ($\log_{10}$)	Recovery of input virus (%)
PRV	130	Yes	≥4.13	18.5
MuLV	80–110	Yes	≥4.72	0.1
BVDV	25–40	Yes	0.4	42.5
HAV	22–30	No	≥3.84	37.5
PPV	18–26	No	≥6.98	102.5

Membrane chromatography devices are single use. Thus, it is important to consider inter and intra lot consistency when validating the devices for contaminant removal and virus clearance. Small scale this matter is simple and relatively affordable for process and product related contaminants. In this case, clearance power is determined by spiking experiments. The goal of these experiments is to demonstrate that the membrane chromatography device removes the spiked contaminant to a specific, consistent and predictable value. Large scale, this is not an affordable option because virus clearance runs are expensive to operate. None the less inter and intra lot consistency data has been generated and is provided in Table 7.

It is important to demonstrate that the clearance power achieved in spiking studies is greater than the amount of contaminant in the feed stream. This also implies that there is a known amount or absence of contaminants in the feed stream and the concentration is consistent to some degree. It is critical to show that there is a margin between contaminant clearance and contaminant in the feed stream. If not there is no proof that the particular contaminant or virus of interest is removed from the product. Product recovery is another goal that is equally important to achieve. This should also be consistent and to an acceptable level (>95%). Ultimately, log reduction values always correlate with a throughput value achieved with the membrane chromatography device. Consider Table 8. log reduction values are shown for each virus at a throughout value of 3000 g/m^2 of Q membrane.

Virus clearance is a complex issue and is covered in depth in another chapter of this text. However, there are some aspects of virus clearance with membrane chromatography devices that are worth discussing here. In brief, virus clearance is defined as the sum of all virus clearance unit operations in a biomanufacturing process. So long as the mode of operation between the unit operations are complementary to each other (CPMP, 1997). Most virus clearance strategies use low pH/solvent detergent; virus filtration and FT-AEX chromatography in order to gain an overall virus clearance value that is acceptable for the process. Each step eliminates virus from product in a different way. Thus, the virus clearance value achieved from each step can be added to the others.

TABLE 7 Virus Clearance LRV Variability Between Membrane Chromatography Lots

	Lot number				
	521083	521283	521183	Ave.	SD
LRV–MVM[a]	6.6	6.73	6.73	6.69	0.08
LRV–Reo III[b]	3.49	3.85	3.67	3.67	0.18

[a] ~9g/L at 600cm/hr, 4.5mS and pH 7.2, 1% MVM Spike.
[b] ~4.5g/L at 450cm/hr, 4.5mS and pH 7.2, 0.5% Reo-3 Spike.

TABLE 8 Virus Clearance Values for Large Scale Processing Runs[a]

Viruses	Size (nm)	Enveloped	LRV run 1	LRV run 2	% Recovery
PRV Pseudorabies virus	120–200	Yes	>5.58	>5.58	100
MuLV: Murine leukemia virus	80–110	Yes	>5.35	>5.52	70
Reo-III respiratory enteric orphan III	60–80	No	>7.00	>6.94	100
MMV: Minute mouse virus	16–25	No	>6.03	>6.03	100

[a] ThroughPut value = 3000 g/m^2 of membrane.

Membrane chromatography devices used in validation studies prove that the mode of operation is adsorption, not size exclusion. During validation studies, a known amount of product flows through a defined volume of Q membrane at linear velocities ~500 cm/*h*. The product is spiked with virus (0.1–1% v/v). Thus, there is a known amount of virus in the load. The membrane is washed with a low salt solution after the entire spiked load is passed over the Q membrane. This ensures the entire product is flushed out of the membrane and product recovery is high. After this, the membrane is striped with 2 M NaCl. This elutes bound virus. Total virus in the load; flow through; wash and elute is determined by biological or molecular assays ($TCID_{50}$ or qPCR). Virus recovery is 100%, if the amount of virus in the load is equal to the amount in the flow through + wash + elute. It is important to note that some viruses are not stable in high salt or are shear sensitive. Some of these particles are destroyed during elution. This reduces recovery values for these viruses. Murine Leukemia virus is one such virus. Recovery for this virus is lower than others used as model virus for clearance trial. However, if this is a concern, consider the size and recovery of Pseudo Rabies virus. This virus is 40% larger than MuLV and its recovery is typically 100%. Evidence that suggests that MuLV recovery is not compromised by size exclusion.

100% recovery is not entirely necessary but does prove that the membrane chromatography device is removing virus by adsorption not retention. This is important to realize if there is a virus removing filter in the same process and both steps are validated for virus clearance. Virus clearance values achieved with a membrane chromatography device that is removing virus by retention should not be added to virus clearance values achieved by virus filtration. The modes of operation are not orthogonal.

Disposability eliminates the risk of carry over contamination and the need for cycling studies. These attributes streamline validation efforts, reducing the amount of time and paperwork. Viruses do carry over in clearance studies that involve cycling of chromatography media (Bose, 2005). After a contaminant, impurity or virus binds to a single use device; they are disposed and will never enter the process again. Cleaning does reduce the risk but it has been shown that contaminants can carry over. As a result compromised batches are possible. This has occurred in the past and will occur in the future. The cause may be variable. However, process engineers and developers can make every effort to reduce this probability. Single use concepts provide an option to at least eliminate one cause.

Re-usable chromatography media should demonstrate that the level of clearance at the end of its lifetime is not dramatically different that the first cycle. This requires the process developer to commit time and resources to cycling studies with and without spiked contaminants. A re-usable device may have great performance without spiked contaminants. This can be up to 100 cycles or more. Validation studies require loading

spiked material during cycle one and the last cycle but there is always the chance that the last cycle will not have the same level of clearance as the first. So, intermediate cycles are spiked to determine up to how many cycles the re-usable device can demonstrate the power to remove contaminants. This can be anywhere between 25% and 100% of the cycles demonstrated without spiked material. Single use contaminant removal devices eliminate such studies. After each run a new device is used. The question of intra and inter lot variability are addressed with thorough validation studies described above and will be discussed in the next section—suppliers ensure quality by provide detailed product validation and lot release data.

A major step forward in the validation process is to show that the membrane chromatography device will consistently remove contaminants/virus from product, regardless or membrane lot number. Since this is a chromatography application, conditions in the mobile phase (pH, conductivity, product concentration) must remain somewhat consistent in order for this to occur. However flow rate can vary, for reasons already discussed.

Implementation is a matter of identifying the correct sized device for large scale production. LRV of contaminants and virus at a given throughput is known. Simply divide the grams of product at process scale by the throughput value. The product of this equation indicates device volume and flow rates that will be used at that scale.

Manufactures provide product validation data upon request and certificates of analysis with each product. Information within validation documents gives detailed account of quality control concept; technical specifications and chemical compatibility/ extractables.

Quality control concept includes a short statement and certification on the supplier's quality management system and its ability to show complete traceability. This section may also include the drug master file number for the membrane. A detailed description of the quality assurance methods is given. This includes how the filter and membrane material are inspected and tested; how protein binding capacity and flow rate for the membrane is characterized; shelf life and how capsules are tested for quality assurance.

Technical specifications demonstrate the mechanism of adsorption and a description of available ligands. Other useful product information, like part numbers and product dimensions, construction and flow pattern are also given here. If the device is autoclavable, this is where detailed directions for this process can be found. Binding capacity for each chemistry and flow rates for capsules are detailed too. Of course chemical compatibility and extractables information is given also.

Certificates of analysis that are supplied with each product ensures the end user that the product meets the specifications for release and quantifies those values.

SUMMARY

Membrane chromatography's track record in validated, large scale biopharmaceutical processes is a reflection of how this industry is responding to bottlenecks surfacing in downstream purification. This technology provides a disposable, cleaning validation free option that shares the same level of contaminant clearance as conventional columns. In other cases membrane chromatography shows promise as a mechanism to rapidly concentrate and purify dilute feed streams. What is difficult to realize but is true, is that these applications, validated or not, consume only a fraction of the time and consumables

as conventional chromatography. Advantages experienced while operating these devices translate directly or indirectly into improved process economy. Smaller footprints, disposability, reduced processing time and ease of use feed directly into reducing cost of goods.

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23

Expanded Polytetrafluoroethylene Membranes and Their Applications

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INTRODUCTION

The unique properties of expanded polytetrafluoroethylene (ePTFE) membrane make it a good choice for a number of pharmaceutical applications. Currently, ePTFE constructions are used for sterile filtration of fermentation feed air, process gases, and tank venting. They are also used in powder collectors and ultralow penetration air (ULPA) filters. The unique properties of ePTFE are also being exploited in a number of new innovative products and technologies. Lyophilization trays, product isolators, and drug delivery devices are just a few of the new areas of interest to the pharmaceutical industry. This chapter will:

- discuss some pertinent properties of PTFE
- explain how PTFE is made into a microporous membrane
- describe the unique characteristics of ePTFE membranes
- discuss the benefits of these characteristics in pharmaceutical applications
- introduce a selection of emerging technologies based on ePTFE membranes

PROPERTIES OF PTFE

Polytetrafluoroethylene or PTFE $(CF_2—CF_2)_n$, commonly referred to by the DuPont trademark Teflon® or the ICI trademark Fluon®, is well known for its chemical resistance, thermal stability, and hydrophobicity. PTFE has these desirable characteristics because of its unique chemical structure, as seen in Figure 1.

PTFE is a simple polymer because it is composed of only two elements: carbon and fluorine. PTFE has a long, straight carbon backbone to which the fluorine atoms are bonded. Both the C–C and C–F bonds are extremely strong. In addition, the electron cloud of the fluorine atoms forms a uniform helical sheath that protects the carbon backbone. The even distribution of fluorine atoms makes it nonpolar and nonreactive. The combination of strong bonds, a protective sheath, and nonpolarity make PTFE extremely inert as well as thermally stable. This explains why PTFE is compatible with

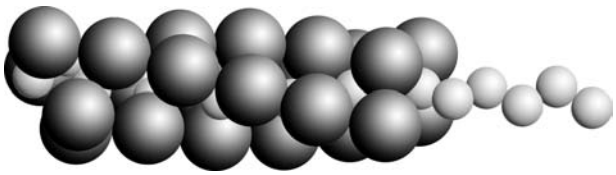


FIGURE 1 Chemical structure of polytetrafluoroethylene (PTFE).

TABLE 1 Physical Properties of Polytetrafluoroethylene (PTFE)

Property	
Structure	— (CF_2CF_2)—$_n$
Surface free energy	18.5 dyn/cm
Melt temperature	327°C
Continuous service temperature	288°C

nearly all the processing and cleaning fluids that are typically used in the pharmaceutical industry, including acids, bases, and solvents.

Because of the nonreactivity and nonpolarity of PTFE, it is difficult for anything to bond to it. This is why PTFE (Teflon®) is well-known as a nonsticking and easy-to-clean product.

Since fluorine is the most electronegative element in the periodic table, it does not want to share electrons with neighboring fluorine atoms. This results in a low surface free energy for PTFE. The lower the surface free energy of a material, the less likely it is to be wetted with higher surface energy fluids such as water. Table 1 summarizes some of the physical properties of PTFE.

In contrast, other polymeric membrane materials have some or all of the fluorine atoms replaced with hydrogen or other elements. This results in weaker bonds and a more polar, reactive molecule. The substitution also increases the surface free energy. Therefore, these polymers are less hydrophobic, less thermally stable, and more reactive than PTFE.

Figure 2 is a chemical compatibility and temperature map, which is a visual way to compare the chemical and thermal stability of various polymers.

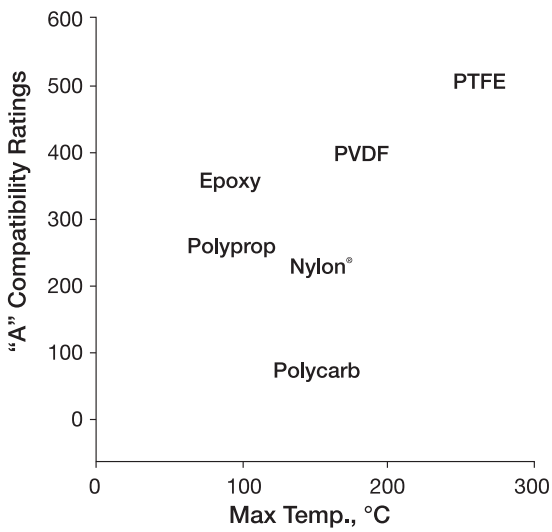


FIGURE 2 Chemical compatibility & temperature map.

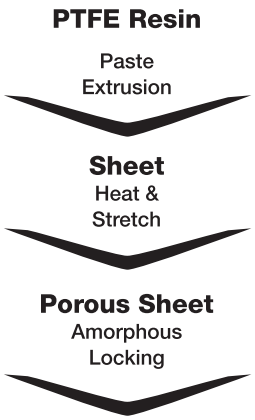


FIGURE 3 ePTFE process flow.

MICROPOROUS PTFE

Since PTFE is chemically inert, thermally stable, and extremely hydrophobic, it is an ideal polymer for some pharmaceutical applications. As a microporous membrane, PTFE is a valuable air-filter medium with high flow rates and filtration efficiency.

A schematic of how ePTFE membrane is made is shown in Figure 3. In general, the process begins with pure PTFE fine powder resin. A lubricating agent is added so that the powder forms a paste and can then be extruded into sheet form. This sheet is heated and expanded under the proper conditions to make a microporous sheet. The structure is stabilized in an amorphous locking step. Though most polymers fracture when subjected to a high rate of strain, expanding PTFE at extremely high rates actually increases the tensile strength of the polymer. Since the lubricating agent is extremely volatile, it is completely removed from the porous structure during processing. The resulting product is 100% PTFE.

Another method used to manufacture porous PTFE is a replication process in which PTFE particles are mixed with burnable material such as paper fibers, then heated to remove the fibers. PTFE can also be made porous by removing a fugitive material such as a carbonate. Because these methods yield products that have lower flow rates and more contamination, they are of little commercial value to the pharmaceutical industry. The rest of this chapter focuses on microporous ePTFE membranes.

Figure 4 shows a scanning electron micrograph of an ePTFE structure that is commonly used in pharmaceutical microfiltration.

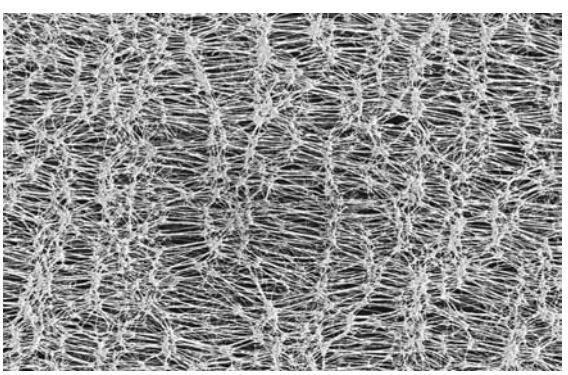


FIGURE 4 Scanning electron micrograph (SEM) of ePTFE.

TABLE 2 Physical Properties of Expanded Polytetrafluoroethylene (ePTFE)

Property	Range
Porosity	1 – 99%
Airflow	2.0 – 15,000 mL/cm^{2a}
Methanol flow rate	1.0 – 10,000 mL/cm^{2b}
Water entry pressure	0 – 350 psi
Isopropanol bubble point	0.1 to > 50 psi
Pore size	0.02 – 40 μm^{c}

[a] Airflow rate measured at 4.88 in. H_2O at 21°C.
[b] Methanol flow rate measured at 27.5 in. Hg at 21°C.
[c] Pore size as correlated to IPA bubble point.

The microstructure is characterized by nodes that are interconnected by fibrils. ePTFE is a single, continuous structure in which all the fibrils and nodes connect. There are no loose ends or particles to be shed or to contaminate a fluid stream. Even though there is a high density of thin fibrils, this structure is high flowing because it also has a high void volume (~85% porous). Table 2 shows that ePTFE has a broad range of physical properties, including pore sizes, flow rates, and water breakthrough pressures.

Membranes can be engineered and optimized to meet the needs of particular applications (Figure 5).

Because ePTFE membranes are extremely microporous and hydrophobic, they retain water droplets, while allowing water vapor to readily pass through as illustrated in Figure 6.

Moisture vapor flows through an ePTFE membrane by either bulk gas flow or diffusion. If the pressure differs across the membrane, gas flows from the high pressure side to the low pressure side. Moisture vapor also diffuses through the microporous structure if humidity or temperature varies across the membrane.

Expanded PTFE membranes are sometimes laminated to materials for additional structural reinforcement. They can be laminated to felts, wovens, and nonwovens made

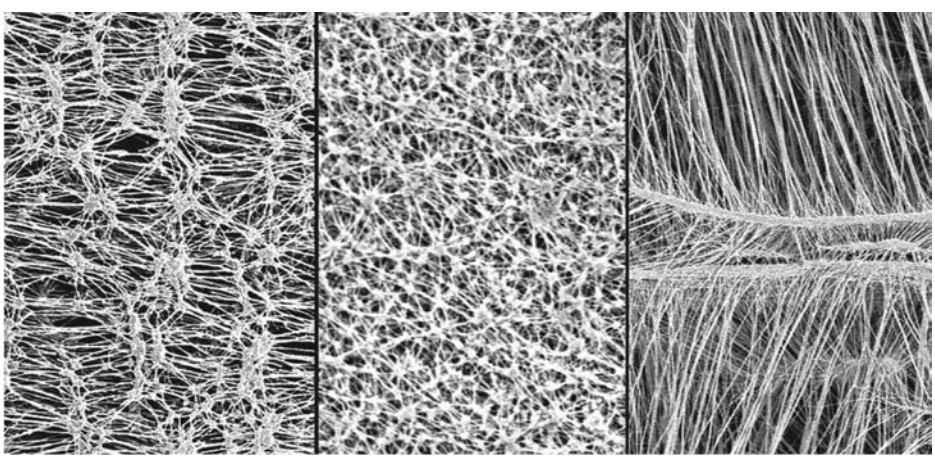


FIGURE 5 Examples of ePTFE structures engineered for specific applications.

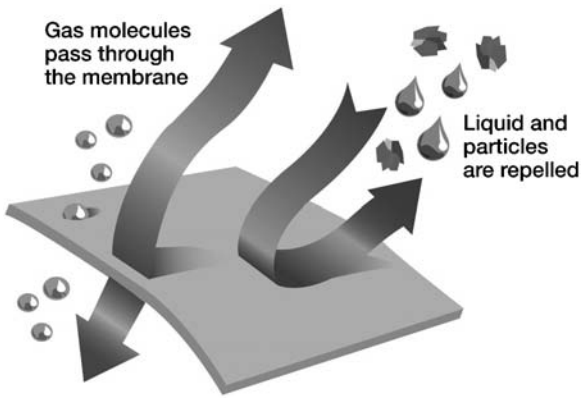


FIGURE 6 Functional characteristics of ePTFE.

from a variety of materials including polyolefins, polyesters, and fluoropolymers. These support layers sometimes function as a drainage layer in filters. Support materials are selected according to the chemical, thermal, and mechanical requirements of the application.

PORE SIZE MEASUREMENT

As discussed elsewhere in this volume, pore size is a relative term, often depending on the application and the test method used to measure it. ePTFE is a highly porous material. Scanning electron micrographs (Figure 4 and Figure 5) make it obvious that ePTFE resembles a tangled forest more than a neatly perforated plate. Though the fibers are well-defined units, they deviate from an ideal circular pore, like almost all porous materials. A common method for quantitatively determining the pore size of membranes including ePTFE is the bubble point method (Figure 7).

In this test, a membrane is wet-out with an appropriate test fluid, such as isopropyl alcohol. Then gas pressure is applied to one side of the membrane. The pressure at which

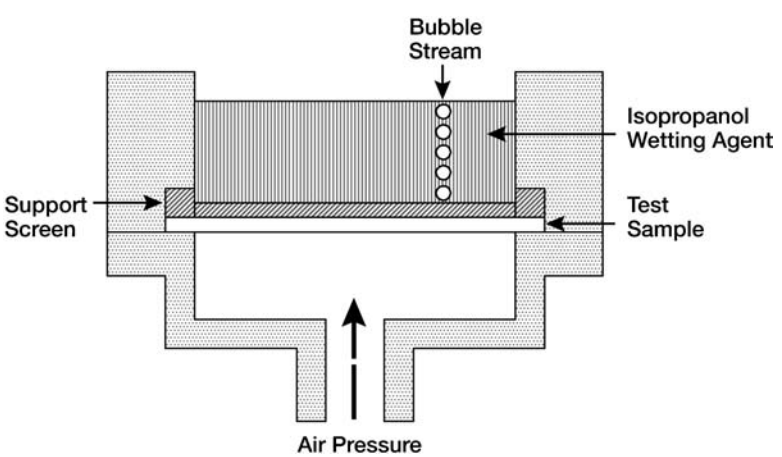


FIGURE 7 Bubble point method for measuring relative pore size.

$$D = \frac{4\gamma \cos\theta}{\Delta P}$$

where
- D = pore diameter
- γ = surface tension of wetting fluid
- θ = contact angle of wetting fluid on filter surface
- ΔP = pressure difference between gas and liquid

FIGURE 8 Bubble point equation assuming cylindrical pore size.

the first steady stream of bubbles (or first measurable bulk gas flow) appears is said to be the bubble point. The inverse pressure–pore size relationship for a cylindrical pore is expressed by the equation in Figure 8.

Note that the contact angle for a wetting fluid like isopropyl alcohol is less than 90°, which keeps the cosθ term positive. For any pore structure other than a cylinder, modifying shape factors must be added to the equation. Since most membranes, including ePTFE, do not have a cylindrical pore structure, a complex interaction of the wetting fluid, membrane, and test gas is actually measured. Therefore, while the general inverse relationship holds that higher bubble point pressures indicate tighter membrane structures (smaller pore sizes), an actual pore diameter cannot be precisely determined from a bubble point measurement.

Water breakthrough or penetration pressure testing of hydrophobic membranes like ePTFE also gives a relative measure of the pore size of a membrane. Water breakthrough pressure is the minimum pressure required to force water through the largest pore of a dry hydrophobic membrane as shown in Figure 9.

The contact angle in this case is larger than 90°, which leads to a negative pressure difference (i.e., the liquid pressure has to be higher than the gas pressure). Every hydrophobic membrane has a unique water breakthrough pressure that depends on the membrane's surface free energy, pore size, and shape. The inverse relationship between pressure and pore size also holds with this test in that tighter structures require higher pressures to force water through the membrane.

Recently, pore size distribution measurements such as liquid porometry and mercury porosimetry have become more common as a means to characterize filtration media. The liquid porometry test is similar to the bubble point test previously described, but instead of measuring only the largest pore as in the bubble point test, the pressure is

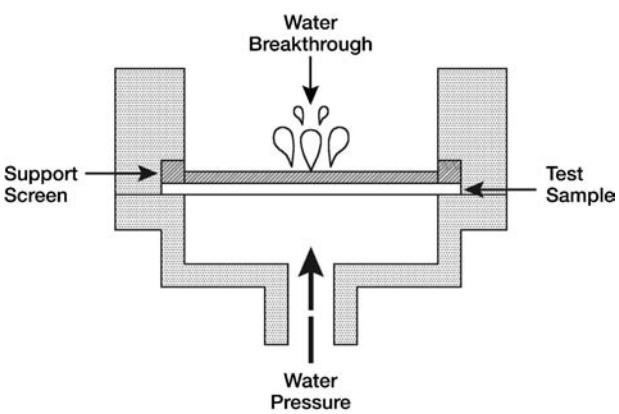


FIGURE 9 Water breakthrough method.

continually increased to open smaller pores. The flow rate through the fraction of open pores at a given pressure is compared to the flow rate through the dry filtration media to assess the fraction of open pores at that pressure. This determines the cumulative volumetric pore size distribution. Mercury porosimetry works similarly to the water breakthrough test. Mercury, a nonwetting liquid, is forced into the porous structure. The pressure is continually increased forcing the mercury into smaller and smaller pores according to the equation in Figure 8. The measured relationship of applied pressure to the volume occupied by mercury leads to the pore size distribution.

Use and Misuse of Pore Size Measurements

Filtration efficiency testing in both air and liquids provides information about the pore structure of a membrane. In aerosol filtration, it is difficult to determine the actual pore size of a membrane because adsorptive effects allow the filter to capture particles that would otherwise pass easily through the structure. Thus, the efficiency test gives a good estimate of the pore size only if the dominant filtration mechanism is that of sieving or size exclusion, which is not necessarily the case for air filtration applications. These tests can be used, however, to compare the relative effectiveness of different filters in a given application.

The true pore size distribution of an air filter does not by itself determine the capture efficiency versus particle size curve, because most particles that can fit through the pores are still captured by other mechanisms to be discussed later. Moreover, practical pore-size measurement methods can only infer pore sizes indirectly and imperfectly. For example, many methods begin by assuming that the pores are cylindrical, but microscopy shows that this is far from true. Thus, particles that are much smaller than the measured pore size of a filter are mostly surface-filtered. On the other hand, a filter's pore-size rating may be claimed based on the size of the smallest particle that is captured, which, however, may vary tremendously depending on how the retention is measured (e.g., air velocity). Air filtration efficiency for an ePTFE membrane having a nominal pore size of 5.0 μm may be better than 99.99% at 0.1 μm. For these reasons, pore size can be a confusing specification for an air filter, and it is often more useful to specify the retention efficiency for the particle size and conditions of interest.

AIR FILTRATION THEORY

Modes of Air Filtration

Air filtration occurs in two ways—surface filtration and depth filtration. Simply put, surface filtration occurs when particles are too large to fit into the pores of a filter and are trapped on the surface of the filter medium. Depth filtration occurs when particles are small enough to fit into the pores but are trapped during their journey through the depth of the filter medium. Therefore, the interaction between the particle size distribution and the pore size distribution determines whether surface or depth filtration occurs.

The Surface Filtration Mode

Air filters sometimes work by excluding large particles from the smaller pores in the filter. This mechanism of particle capture is called sieving. Particles that are larger than the pore diameter do not enter into the depth of the filter. In air filtration, surface filtration

mode is actually less common than depth filtration. It is only in this relatively uncommon mode of filtration that pore diameter is important. ePTFE membranes are unusual in that they often provide good surface filtration due to their small and consistent pores.

In one sense, no element of chance is involved in surface filtration. If a particle is bigger than the pores, it is collected. But in another sense, chance is still involved, because the pore sizes all have some statistical distribution. If there is a tiny population of large pores, then some of the large particles are carried through those pores. If a particle enters the depth of a filter because it is not surface-filtered, depth filtration remains available to help capture it.

The Depth Filtration Mode

In depth filtration, particles small enough to enter the filter structure are collected by chance interactions with the fibers of the filter. If the structure of the filter is the same at all depths, as it is in most filters, there is an exponential decay in the particle population as the air passes through the depth of the filter. This filtration mode is consistent with a constant probability of collection within the next increment of depth for a particle that has survived the trip so far.

Depth filtration is sometimes incorrectly considered the opposite of absolute filtration. It is easy to demonstrate that the penetration through some depth filters may be 10^{-20}, 10^{-50}, or less. A penetration of 10^{-20} cannot be measured, and a penetration of 10^{-50} has no physical significance at all (being about one atom's worth of the earth's mass). Thus such a depth filter at this efficiency level can be considered absolute.

Depth filtration is a two-step process: transporting the particles to the internal fibers of the filter and attaching them to the surface of the fiber. Briefly, all depth filtration mechanisms rely on van der Waals forces, electrostatic adhesion, or both to hold particles to filter fibers once the particles contact the fibers. However, mechanisms differ in why particle–fiber contact occurs. Depth filtration works by several particle capture mechanisms, unlike surface filtration, which works only by sieving. These mechanisms are discussed in more detail elsewhere in this book and are thoroughly discussed in Hinds (1982). In the impaction mechanism, large fast-moving particles hit fibers because their momentum overpowers the airflow deviating around the fibers. In the interception mechanism, relatively incompressible particles barely touch the fibers, while nearby airstreams compress together around the particles to slip by the fibers. In the diffusion mechanism, small particles move vigorously during collisions with individual air molecules, increasing random contact with fibers. Impaction and interception work better on larger particles, whereas diffusion favors small particles. Therefore, extremely large or small particles are collected easily, whereas an intermediate most-penetrating size of particle is difficult to collect. Filters made of finer fibers have a smaller most-penetrating particle size than filters made of coarser fibers.

The fibrils in ePTFE membranes designed for filtration are smaller in diameter than those in any other commonly used filtration medium. Many fibrils in ePTFE are as small as 0.01 or 0.02 μm, with a median diameter of 0.05 or 0.1 μm. In comparison, most fibers in micro-fiberglass paper are about 1 μm, and fibers in filtration textiles are about 10 μm or more (other membranes may have fibers for which the diameter is hard to define, though numbers around 0.1–1 μm are possible). From the viewpoint of the air filtration theorist, ePTFE is nearly ideal because of its random mesh of fine fibers distributed almost perpendicularly to the airflow, with a high void fraction. The most penetrating particle size is around 0.07 μm, depending on air velocity. This makes ePTFE filters very efficient

on the basis of total penetrating particle mass, as a 0.07 μm particle contains nearly 80 times less material than the 0.30 μm particle that is most penetrating for typical filters such as High efficiency particle air (HEPA) filters. (Remember that the volume of a sphere varies as diameter cubed). Moreover, very fine fibers offer little resistance to passing air, so good capture efficiency for ePTFE comes at little expense in air pressure drop.

Pressure Increase and Cleaning in the Two Modes

The most significant implication of using surface versus depth filtration is the location of captured particles. In surface filtration, the particles accumulate in a thin, dense layer right at the surface of the filter, whereas in depth filtration they accumulate gradually throughout some depth. The advantage of depositing the particles right on the surface is that they can be removed more easily, and the filter can thus be cleaned and recover its initial airflow. The advantage of depositing the particles throughout the depth is that the particles are diffused through a discontinuous region, which still has a large fraction of open space. Airflow is thus more easily maintained without removing the particles in depth filtration.

It is useful to consider three different cases of loading of a filter. In the lightest particulate loadings, such as those experienced by high-purity, point-of-use filters or recirculation filters sealed inside computer disk drives, the effect of particle loading is unimportant, because hardly any particulates are present. In this case, it is unimportant whether surface or depth filtration dominates, and filters are chosen based on other criteria.

When particle loading is very heavy and rapid, it is necessary to clean the filter frequently, perhaps thousands of times during its lifetime. An example would be bag house or cartridge collectors for food or pharmaceutical powder collection. In this case, surface filtration is crucial, making it easy to remove particles thoroughly in each cleaning cycle. ePTFE membrane filters are very useful in these applications because they are easy to clean. An additional benefit of ePTFE is the minimal adhesion that exists specifically between polytetrafluoroethylene and particulates (the same benefit that favors Teflon® frying pans).

In the third case, between these two extremes, the particulate loading is low enough to make cleaning unnecessary, but high enough to limit usable lifetime. In this case, surface filtration can be disadvantageous, especially if the particulate is oily and tends to wick into a continuous sheet. In this case, membrane filters (including ePTFE membranes) acting as surface filters require a prefiltering layer to perform depth filtration, especially for larger particles that constitute a great majority of the particulate mass. Such a prefilter layer with an efficiency of only 95% for all combined mass extends the life of the membrane 20-fold, making this strategy a successful solution. Interestingly, the classical dioctylphthalate (DOP) smoke efficiency test falls into this class for ePTFE membranes if excessively high loadings are used, although the loadings used in the TSI Model AFT8160 filter tester do not cause a problem.

Improved Efficiency During Cleaning and Lower Pressure Drop

Filters used for extremely high particulate loadings must be cleaned repeatedly. Yet some filters rely on particles bridging and filter cakes forming over openings in the nonmembrane filter media to help capture other particles. During cleaning, these particle bridges are disrupted, and particle retention efficiency is reduced. Relative to

most filters of this type, ePTFE filters have excellent retention efficiencies, even during the cleaning cycle. The ePTFE membrane on the surface serves as a permanent size-exclusion layer. However, this permanent layer does not inhibit a pressure drop like a filter cake.

Summary of Air Filtration Theory

Compared to most other air filtration media, ePTFE membranes are unusual in several ways:

- Their high density of extremely fine fibrils provides better depth filtration efficiency.
- High porosity and fine fibrils offer little resistance to airflow.
- Small and consistent pores often provide surface filtration.
- Cleaning of surface-filtered particles is very easy.
- Filter efficiency is still high during and immediately after cleaning.
- They have high air filtration efficiencies combined with low pressure drop.

STERILIZATION

Materials used in pharmaceutical manufacturing can be sterilized by numerous methods, including dry heat, steam, ethylene oxide gas (ETO), or ionizing radiation. Because of PTFE's thermal stability, ePTFE can be repeatedly thermally sterilized under typical autoclave, steam-in-place, and dry-heat oven conditions. Also, because PTFE is chemically inert, it is unaffected by ETO sterilization or cleaning solutions.

Steam and cleaning solutions can wet-out the pores of a hydrophilic or less hydrophobic polymer. The liquid in the membrane pores can block gas flow as well as create an environment in which microorganisms can grow. Since PTFE is extremely hydrophobic, using ePTFE minimizes these risks.

Although PTFE exhibits extraordinary resistance to chemical attack from a wide range of chemical species, it has only limited resistance to ionizing radiation. Ionizing radiation causes chain scission of the carbon bonds in the backbone of the helical PTFE molecule. This not only reduces molecular weight but also reduces the ultimate tensile strength and ultimate elongation. This decrease in physical properties is observed at relatively low levels of radiation; significant decreases are observed at less than 0.5 mRad. Because of the decrease in physical properties, ionizing radiation is not a preferred method of sterilizing PTFE.

In some applications, components containing ePTFE still need to be sterilized with radiation. Properly designed and supported ePTFE laminates have been radiation sterilized and successfully used as hydrophobic vents and sterile barriers such as transducer protectors, intravenous spike vents, and other medical components for a number of years. In these products, the materials dependably and reliably support the ePTFE to prevent degradation of physical properties. The microstructure, physical dimensions, and performance of the laminate are not changed after radiation sterilization. Physical conditions that cause an ePTFE membrane to fail after radiation sterilization include excessive elongation, puncture on sharp protrusions, and concentration of stresses in limited areas.

FERMENTATION FEED AIR AND EXHAUST GAS FILTRATION

Large volumes of sterile air and gases are required as a raw material in aerobic fermentation reactions. These gases require filtering to remove organisms and particulates. Exhaust gases are filtered to prevent the fermentor from contaminating the environment as well as to prevent the environment from contaminating the fermentor. During their service life (typically more than one year), these filters must undergo repeated steam sterilizations without blinding or losing their integrity. Filter failure is extremely costly in terms of product yield, product quality, maintenance, and downtime.

Historically, packed towers were used for these types of filtration. While packed towers are better than no filtration, they have several disadvantages—they are relatively inefficient, expensive to install and operate, wasteful of space, hydrophilic, and impossible to integrity test. Cartridges made of glass fiber and borosilicate glass fibers are more efficient than randomly packed towers, and they also save space. However, users of membrane filters containing ePTFE are realizing significant improvements in filtration efficiency, pressure drop, hydrophobicity, and integrity testability (Figure 10).

Because ePTFE membranes have a narrow pore size distribution, they retain particles and organisms more efficiently than nonwoven depth filtration media. Since ePTFE is extremely hydrophobic, (*i*) it remains hydrophobic through repeated sterilizations, (*ii*) it resumes its presteam pressure drop with a minimal post-steaming blowdown, and (*iii*) it is not susceptible to grow-through.

In addition to the benefits of improved reliability and efficiency, using ePTFE membrane filters is quite beneficial economically for the following reasons:

- *Lower Capital Cost*: Capital and replacement costs are further reduced because fewer filters may be required for a desired flow rate. The installation and capital costs of heat-tracing filter housings are also reduced.
- *Lower Energy Cost*: The lower pressure drop of ePTFE filters results in significant energy savings in compressor operation.
- *Reduced Blow-Down Time*: Since ePTFE is extremely hydrophobic, the time it takes to blow the filter dry is significantly reduced relative to other, less hydrophobic polymers such as polyvinylidene fluoride.

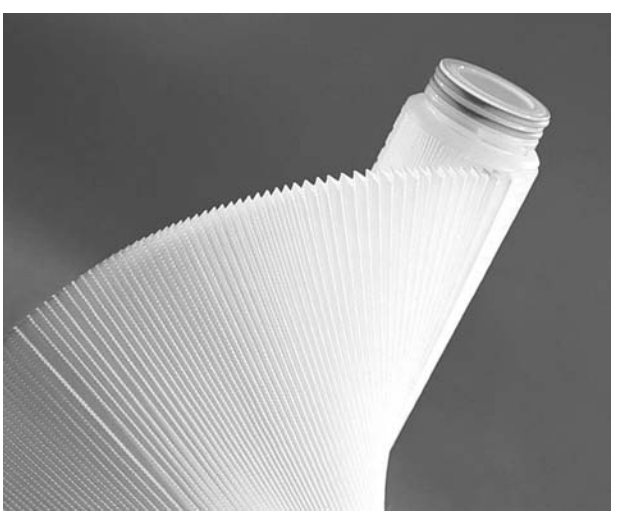


FIGURE 10 Filter cartridge containing ePTFE.

- *Lower Contamination Cost*: Studies have shown that contamination rates are 50% lower in fermentors with hydrophobic cartridge filters than in a control group of fermentors with packed towers

The contamination, energy, maintenance, and productivity cost savings realized by using ePTFE filters can be calculated to determine the total annual cost savings. The payback period for converting to hydrophobic filters is often less than one year.

PROCESS GAS FILTRATION

Several gases, including nitrogen and oxygen, are used in biopharmaceutical manufacturing and finishing. These gases must be bacteria- and particulate-free. ePTFE membranes are ideal for these applications for the same reasons as previously discussed.

VENT FILTERS

Tanks must be vented to allow gas to exit during filling and enter during emptying. If a tank is not vented, or if a vent becomes blocked, the tank can either explode or implode. Even if the filter is only partially blocked, productivity is reduced, because it takes longer to drain the tank. Therefore, a vent filter must allow gas to flow sufficiently to equalize pressure while preventing contaminants from entering the tank.

Autoclaves and lyophilizers typically have vent filters to ensure that the air coming in after sterilization is sterile and particle-free.

Once again, filters made of ePTFE are ideal for these types of applications. The filters allow for a high flow rate at a low pressure drop. These filters can be regularly tested to verify their integrity and their installation. Since they are hydrophobic, ePTFE filters do not "blind" from repeated steam sterilization or incidental contact with aqueous solutions.

LYOPHILIZATION

Many biopharmaceutical products, such as vaccines and monoclonal antibodies, are unstable in aqueous solutions over time. Their shelf life can be enhanced by drying, but many of these substances are also unstable by nature when exposed to heat. Lyophilization, or freeze-drying, allows these pharmaceutical products to be preserved with minimum degradation. During lyophilization, the product is first frozen to below its eutectic or glass transition temperature, usually in the range of $-40^{\circ}C$ to $-60^{\circ}C$. A vacuum is applied and energy is input to create the proper conditions for sublimation. Removing moisture through sublimation, in which a solid (usually ice) is transferred to the gaseous phase directly, provides a low-heat input method for preserving and extending the shelf life of valuable pharmaceutical products that otherwise become unstable during processing or storage.

Single-dose pharmaceutical products are typically freeze-dried in glass vials. The conventional method for freeze-drying in vials uses a split-bottom stopper partially inserted in the top of the vial, leaving an opening throughout the freeze-drying cycle, so that air and moisture vapor can escape (Figure 11).



FIGURE 11 Vapor path during freeze-drying cycle with conventional split-bottom stopper.

Because most of these products are parenteral (injectable) and heat-sensitive, they are usually manufactured in aseptic processes and cannot be terminally sterilized. While environmental controls and other precautions are used to prevent contamination, the vials are open throughout the process and encounter several inherent risks, including:

- contamination of the product during transport to and loading of the lyophilizer
- contamination of the product during the lyophlization cycle
- cross-contamination between containers in the lyophilizer
- product loss and difficult cleanup due to entrained powder
- worker exposure to toxic compounds being lyophilized

Inserting an ePTFE membrane in an overcap minimizes these risks by isolating the content of the container from the external environment. The product isolator protects and contains the product during processing using standard glass vials and stoppers (Figure 12).

Figure 13 shows the following steps for attaching, using, and removing a product isolator:

1. Fill the vial in a controlled environment.
2. Insert the stopper in the down position.
3. Apply the product isolator in the down position.
4. Raise the product isolator to the venting position. This automatically raises the stopper, creating an annular space between the stopper and vial wall. This annular space provides a path for water and other vapors to escape during sublimation. Transfer vials to the freeze-dryer, and run the freeze-dry cycle.
5. At the end of the freeze-dry cycle, the freeze-dryer shelves collapse, which automatically seat the product isolator and stopper. After lyophilization is complete, remove the vial from the freeze-dryer.
6. Remove the product isolator, while keeping the stopper seated.
7. Crimp the stopper on the vial.

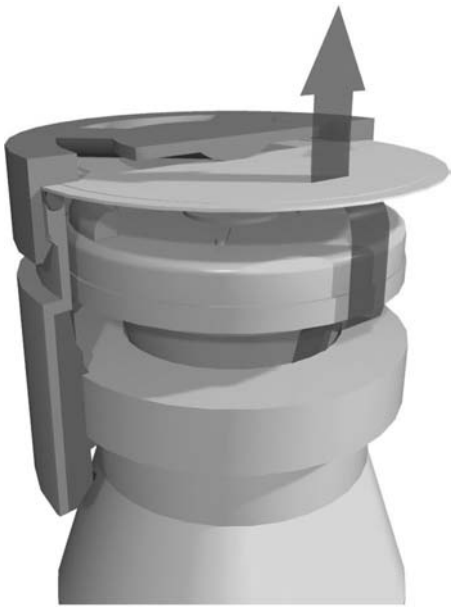


FIGURE 12 Vapor path during freeze-drying cycle with product isolator.

The ePTFE membrane allows both diffusion and bulk flow of moisture vapor through it, ensuring that the sublimate has an exit path from the package throughout the freeze-dry cycle. At the same time, the ePTFE vent not only contains the dried product within the package but also prevents foreign particles from entering the package. Also, since ePTFE is extremely hydrophobic, it retains aqueous solutions during loading and drying.

The ePTFE membrane is an effective sterile barrier that protects the product from bacteria, viruses, and other contaminants, allowing manufacturers greater freedom in facility design. Since the product is in a closed, integral package after liquid filling, it can be transported between sterile areas or into nonsterile areas without risk of contamination. The thermal resistance of ePTFE allows it to withstand both freeze-drying and autoclave conditions. Likewise, ePTFE is chemically inert, so it is not likely to react with the product.

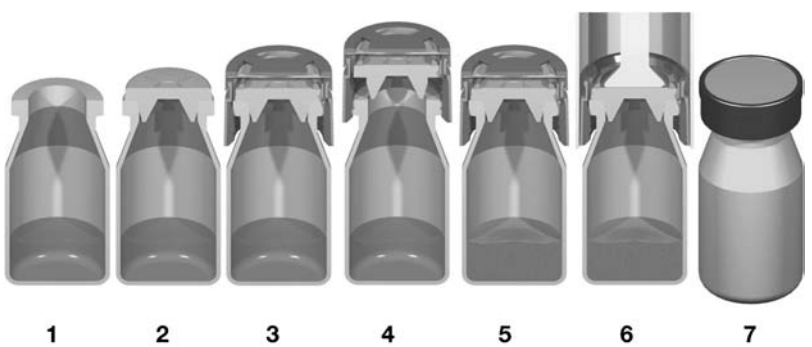


FIGURE 13 Process for attaching, using, and removing product isolator.

In a series of experiments, scientists have found high cross-contamination rates while using conventional split stoppers in glass vials. Barbaree and Sanchez (1982) investigated cross-contamination rates from vial to vial in a freeze-dry run. Vials were filled with either of two bacteria, and the number of times these bacteria traveled from one vial to another during a standard freeze-dry run was counted. The study found cross-contamination rates to be as high as 80% of vials per run. The Barbaree study was repeated and expanded upon by Lange et al. (1997). This study found cross-contamination rates for vials with conventional stoppers to be as high as 91% of vials per run. The Lange et al. study also used two indicator organisms to track the path of cross-contamination but added a new component to the investigation. It compared the incidence of bacterial escape and cross-contamination rates for conventional split stoppers to that for fully seated stoppers with an ePTFE vent.

The Lange et al. study found that in all cases, the indicator organism that contaminated vials with conventional stoppers came from other vials with conventional stoppers. On the other hand, no bacteria were ever found to have either escaped from or entered into the vials with the fully seated ePTFE-vented stoppers. In each run, the incidence of cross-contamination by indicator organisms in the vials with ePTFE-vented stoppers was 0%. These results indicate that ePTFE-vented stoppers are an effective barrier to product contamination.

In recent studies, vials with the isolators and stoppers in the raised position were subjected to a challenge concentration of 1.9×10^9 CFU/mL of *Bacillus atrophaeus* for 60 min followed by a four-hour soak. Following the exposure and soak, the vials were incubated for seven days at 30–35˚C. None of the samples exhibited growth. These studies show that even under extreme conditions, the product isolator with ePTFE membrane is a sterile barrier.

To increase the stability of biopharmaceuticals, bulk intermediates are often freeze-dried as well. These products have very similar needs and risks as those discussed for vials. An ePTFE membrane can also be attached to trays and containers to provide the same product containment and contamination control as discussed for the vials. Figure 14 shows examples of products currently being used by pharmaceutical companies.

The application of vented freeze-dry packaging takes advantage of several unique properties of ePTFE, including sterile barrier performance, temperature resistance, and permeability to moisture vapor. These same principles simplify manufacturing for both single-dose and bulk pharmaceutical products.

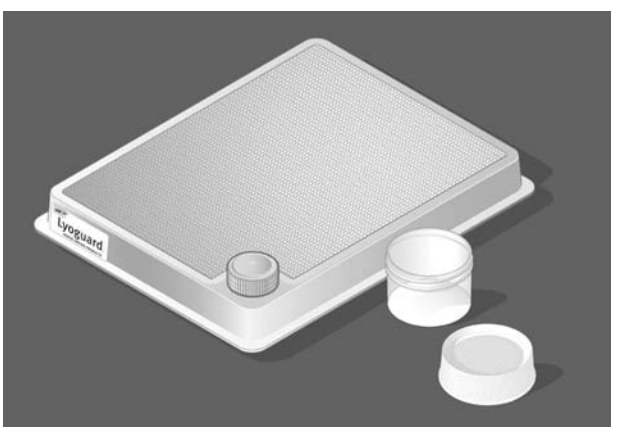


FIGURE 14 R&D and production-scale lyophilization products with ePTFE membrane.

DRUG DELIVERY DEVICES

The growth of non-invasive drug delivery systems is driving the need for innovative and multifunctional drug delivery devices and components. These systems enhance patient compliance and effectively deliver drugs including proteins, peptides, and biologics, as well as traditional pharmaceuticals.

One of today's key challenges in drug delivery device technology is providing accurate dosage while maintaining drug sterility. This has become more of a challenge for device manufacturers as the industry moves towards alternative drug delivery systems. Transmucosal delivery—the delivery of drugs via the eyes, ears, nose, and throat—is expected to grow significantly over the next 10 years.

Many drugs delivered non-orally, such as vaccines, nasal sprays, and eye drops, contain preservatives to provide sterility and extend shelf life. Unfortunately, since many preservatives are irritants and cause adverse side effects, significant efforts are being made to develop unpreserved formulations. Without preservatives, drugs are more susceptible to contamination and require better protection (Figure 15).

A closed system with no incoming contaminated air is the best approach for preservative-free drug delivery. This approach is used for single-unit dose devices, but as the transmucosal drug delivery market grows, the drive for lower-cost, multi-unit dose devices is increasing. In multi-unit dose containers, pressure must be equalized to deliver the drug accurately. Thus, air must be allowed to move in and out of the device. Using 0.2 μm ePTFE membrane filters along with a mechanical one-way closure (such as a check valve) has become a common solution for packaging design of preservative-free formulations and transmucosal delivery systems (Figure 16).

The ePTFE filter allows for accurate dosage and pressure equalization of the drug delivery device while filtering the bacteria- and virus-laden aerosols from incoming air. The ePTFE filter's hydrophobic properties also repel the liquid drug contained in the device, allowing air to flow freely in a liquid-tight container.

The use of ePTFE membrane filters for drug delivery offers many benefits. ePTFE is very low in extractables, is non-particulating, and is chemically inert. It is therefore unlikely to contaminate the drug or react with it throughout the packaged life of the product. It is naturally hydrophobic, and its thermal resistance allows for most sterilization methods to be used.



FIGURE 15 Unfiltered drug delivery device.

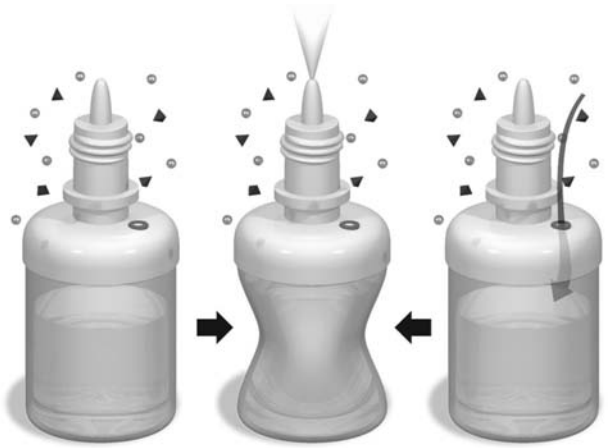


FIGURE 16 Drug delivery device with ePTFE vent and one-way closure.

ePTFE membrane filtration media easily integrate into drug delivery devices. This media can be insert-molded, heat-sealed, or ultrasonically sealed into plastic. Various configurations are offered in which the ePTFE is molded into a separate plastic component and available for insertion into a drug delivery device. Molded components (Figure 17) can easily be compression-fit, spun-welded, or snap-fit into the device, making them a useful and convenient addition to any drug delivery device.

POWDER COLLECTION AND CONTAINMENT

Many pharmaceutical products—whether antibiotics, buffers, antacids, vitamins, or other specialty products—exist in powder form. Typically, these powders are downstream in the purification stages or packaging operations. Separation of the powder from the gas stream requires filtration that must satisfy two requirements:

- prevention of loss of product
- prevention of exposure of product to the operators and to the environment



FIGURE 17 Molded components with ePTFE vents.

Some areas requiring air filtration during processing of pharmaceutical bulk powders are spray drying, fluid bed drying and granulating, tablet coating, milling and grinding, mechanical and pneumatic conveying, silo and bin venting, blending, packaging, and nuisance dust collecting (or local ventilation). The filtration units may actually be contained in the processing equipment or may be downstream in the form of a powder collector. The filter elements used in these applications are typically filter bags or pleated filter cartridges (Figure 18).

Since ePTFE membranes are chemically inert, highly efficient, non-shedding, hydrophobic, and nonsticking, they are an ideal match for many of these applications.

In a powder collector, the air passes through a filter that retains particles and lets clean gas pass through. When there is significant powder buildup on the filters, the powder begins to restrict the flow through the system. The filters then go through a cleaning cycle during which the filters are pulsed with compressed air or mechanically shaken to release the powder, which typically falls into a hopper where the solids are collected.

Conventional media for these pharmaceutical applications typically consist of a felt or woven fabric, such as polyester felt, Nomex® felt, or woven nylon, and they rely on the presence of a primary powder layer or pre-coat to operate efficiently. Media composed of ePTFE membranes are the most efficient, cleanable media in the fabric filtration industry. Collection efficiencies may exceed 99.97% at 0.3 μm from the moment the system starts up. Because all of the product being filtered is captured on the smooth membrane, product holdup decreases and thus minimizes potential product degradation and carryover. While fibers from conventional fabric filters sometimes contaminate the product, this is not possible with ePTFE membrane media, because the product is collected on the membrane side and does not come into contact with the material that supports the membrane. With the increase in demand for processing units with product containment systems, there is an even greater need for an efficient non-linting, FDA-acceptable material to be used as filters in these units.



FIGURE 18 Filter bags and pleated filter cartridges.

In addition to preventing fibers from contaminating the product, the ePTFE membrane has nonstick characteristics that allow the powder to release easily and require less frequent backpulsing. Since particulate matter is not held up within the medium, as with conventional media, valuable product is not lost when the filter is cleaned or discarded. Rather, the powder is recovered as useful product.

Laminates composed of ePTFE membrane offer similar benefits in the pleated cartridge construction. Recently, cartridges have gained popularity because they provide more filter medium in a given space, which ultimately reduces capital costs, decreases the amount of time required for change-outs, and thus decreases downtime. A typical medium for conventional filter cartridges is cellulose paper. The disadvantage of this media is that cellulose has poor resistance to moisture and poor cake release on sticky powder, and it is inefficient on very fine particulate unless precoated. Cellulose media often plug quickly (Figure 19).

Since PTFE is inherently hydrophobic, filters constructed with ePTFE-membrane laminates recover well from moisture upsets. After the moist cake dries, the backpulsing releases the cake and operates as it did prior to the moisture upset. This hydrophobic nature also allows these filters to be rinsed off with water for cleaning. Since PTFE is extremely inert and thermally stable, ePTFE is well-suited for processing and cleaning fluids typically used in the pharmaceutical industry, including acids, bases, and solvents.

ULTRALOW PENETRATION AIR AND HIGH EFFICIENCY PARTICLE AIR FILTERS

High efficiency particle air filters represent a class of air filters that remove greater than 99.97% of airborne particles having a diameter in excess of 0.30 μm. Although the terms HEPA and ULPA have become standards with specific definitions, they originated as marketing terms for highly efficient, low resistance air filters. These filters were originally used in nuclear heating, ventilating, and air conditioning (HVAC) systems but have since been adopted in the pharmaceutical industry as well as the food, electronics, and other

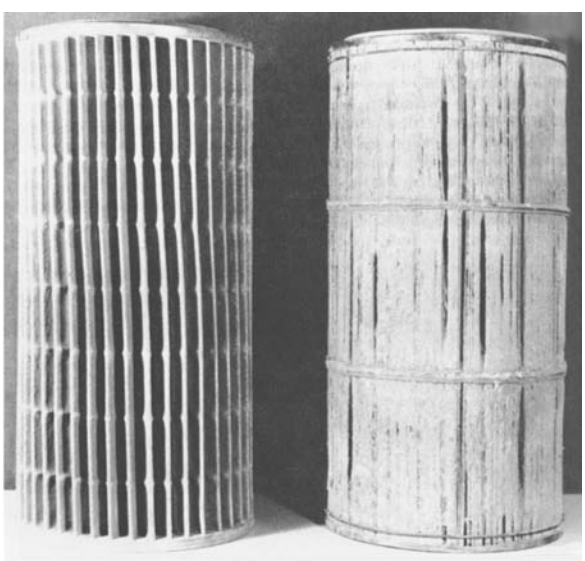


FIGURE 19 ePTFE (*left*) and cellulose (*right*) cartridges exposed to the same environment.

industries. Use in the pharmaceutical industry has primarily been in HVAC and other air-handling systems, like aseptic processing areas, clean benches, hazardous materials hoods, and barrier isolation systems. Little has changed in the technology of these types of filters since their invention. Basically they have been pleated micro-fiberglass paper filters adhered to a frame. Pleat depths range from less than 1 inch to 12 inches. Frames are typically rectangular or circular and made of aluminum or stainless steel.

The major problem with traditional micro-fiberglass HEPA filters is that they have a relatively fragile filter medium. Not only are they easily damaged through normal handling and have limited shelf life, but the rigors of many pharmaceutical applications can harm them. The medium consists of many small glass fibers held together with an acrylic binder. Any breakdown of the binder or fiber causes leaks or shedding. Damaging agents include handling; in-place contact; vibration; humid environments; exposure to chemicals such as cleaning agents, sterilizing agents, or process chemicals; elevated temperature; and age. Disposal of filters that are contaminated with hazardous materials also presents a problem. To circumvent these problems, zones between the clean space and the HEPA filter are usually covered and not subjected to full cleaning or sterilization. Also, filters must be checked for leaks, typically every six months, and are frequently replaced.

The newest innovation in HEPA and ULPA filtration is the use of an ePTFE membrane as the filter medium (Figure 20).

These filters are inherently chemical-resistant, hydrophobic, easily cleaned, and very strong. They have filtration efficiencies exceeding 99.99999% at a most-penetrating particle size of 0.070 μm. The high strength of the medium, combined with the other properties, makes HEPA filters made of ePTFE an excellent choice for many applications, including cleanrooms, barrier isolators, aseptic processing areas, sterility testing areas, and anywhere else where cleanliness, sterility, and contamination control are needed. The filter medium can be exposed to cleaning and sterilizing agents, including water and steam, without breaking down. These filters can be subjected to handling and other physical stresses and still maintain their integrity. In some cases, the filters may be cleaned and reused.

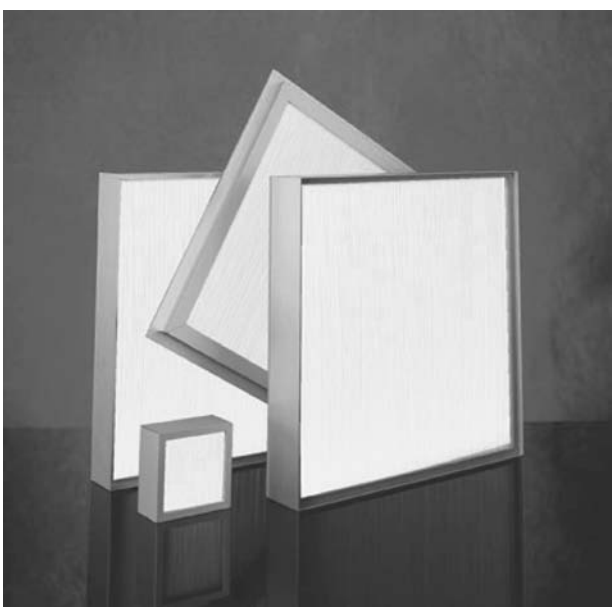


FIGURE 20 ULPA filters with ePTFE membrane.

HEPA filters are checked for leaks at the factory and in use. This is done by challenging the filter with particles and using a particle counter or photometer to detect areas of high penetration. One concern in using ePTFE membrane filters is the use of DOP, Emery 3004, or other oil-based particle challenges. As previously mentioned in this chapter, these oil mists can wick into the filter medium and prematurely hinder airflow. Fortunately, there are many feasible solutions to this problem. As mentioned earlier, a 95% prefilter removes enough mass of oil to prevent dampening of the airflow, leaving the HEPA filter to accomplish the fine, final filtration. Other particle challenges are acceptable and commonly used in the electronics industry. Polystyrene latex (PSL) spheres provide a solid challenge that does not overload the filter. Recent tests have demonstrated that leak location and sizing can be accomplished equally well using PSLs or DOP. Because DOP is a suspected carcinogen, its use is diminishing.

FILTERING LIQUIDS

Because ePTFE membrane is chemically inert and has high flow rates, it is well-suited for various liquid filtration applications. Low surface tension liquids such as alcohols (e.g., methanol or isopropanol) or solvents (e.g., methyl ethyl ketone) can readily wet ePTFE membranes. In these types of applications, no additional prewetting steps are required.

On the other hand, fluids with higher surface tensions such as water do not flow through hydrophobic membranes like ePTFE at normal operating pressures. If a hydrophobic membrane is used for an aqueous filtration, it must first be prewetted with a lower surface tension liquid such as isopropanol. Once properly wetted, aqueous solutions can be filtered through ePTFE membranes. Depending on the application, the prewetting solution can be flushed from the filter with water or with the aqueous solution to be filtered. Prewetting ePTFE membranes is done extensively in the semiconductor industry.

Since prewetting is sometimes inconvenient, a water-wettable or hydrophilic ePTFE membrane is desirable. The surfaces of ePTFE membranes can be modified to render the membranes hydrophilic.

CONCLUSION

Expanded polytetrafluoroethylene membranes are valuable in a number of pharmaceutical filtration, venting, and product collection and containment applications. PTFE is inherently inert, thermally resistant, and extremely hydrophobic, and PTFE can be engineered into a highly retentive and high-flowing microporous membrane. These characteristics make ePTFE membranes the filtration media of choice in gas filtration and venting applications. These desirable characteristics of ePTFE are also being exploited in many new pharmaceutical applications, such as lyophilization trays, product isolators, and drug delivery systems. Because of the unique properties of ePTFE membranes, they are a good choice for these as well as other challenging filtration, separation, venting, and containment applications.

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Air Filtration Applications in the Pharmaceutical Industry

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INTRODUCTION

Many applications exist for air and gas filtration in the pharmaceutical industry. The filtration required can be to the 0.2 μm (sterilizing) level or to a coarser filtration level for particulate removal. Some air applications for which sterile filtration is employed are: fermentor inlet air, fermentor vent gas, vents on Water for Injection tanks, and vacuum break filters on autoclaves and lyophilizers. In Fermentation alone there are five major categories. These include antibiotics, organic acids, amino acids, vitamins, and enzymes and other proteins. Coarser filtration can be employed for particulate removal in which the coarse filter acts as a pre-filter to the sterilizing grade filter. Other applications employ coalescers as prefilters for the removal of liquid droplets, such as oil or water.

This chapter provides information on the applications for air filtration in the pharmaceutical industry and is divided into the following sections: the types of air filters used in the industry, specific air (or gas) filtration applications, recommendations and considerations for proper usage of membrane filters in the applications, and new technology in cartridge air filtration.

MECHANISMS AFFECTING GAS FILTRATION

Regardless of the filtration system used (e.g., packed towers or cartridge filters), the manners in which particles are captured and removed from an air or gas stream are the same. The removal efficiency of filtration media is dependent upon the following mechanisms: (1) direct interception by the fibers, (2) inertial impaction, (3) Brownian motion or diffusional interception, and (4) electrostatic attraction between the fibers and particles (Bruckshaw, 1973).

The filtration mechanism for direct interception is a sieving action that mechanically retains the particles. The filter acts as a "screen" which stops particles that are larger than the pores. Direct interception is independent of face velocity and any other mechanism, and involves particles with diameters larger than the pores of the filter.

Inertial impaction refers to capture of particles due to the inability of the particle to deviate from the change in the fluid flow path because of inertia. This can result in the

retention of the particle by the fibers. As the face velocity increases, the probability of inertial impaction increases. This effect is greatest for particles with larger diameters (typically larger than 1 μm in diameter) or with high densities.

Brownian motion or diffusional interception applies to very small particles (typically less than 0.3 μ in diameter) at low face velocities. When air molecules are in a state of random motion, small particles suspended in the gas stream can be struck by moving air molecules and displaced. The movement of particles resulting from molecular collisions is known as Brownian motion. This phenomena can increase sideways movement of the particles and increases the probability of capture of the particle by the fibers on either side of the fluid path.

In dry air and gas streams, electrostatic charges can build up on the filter matrix. For some materials this effect can be enhanced by surface modification. This electrostatic charge can attract a particle to the surface. An electrostatic attraction can exist between the fibrous material in the depth filter or the membrane filter and the particles in the air stream. This can enhance the ability of the filter to remove particles, all particles including particles much smaller than the pore size rating of the filter medium.

AIR FILTERS—PAST AND PRESENT

Historically, packed towers were the first air filters used by the industry for air sterilization. As technology has advanced, cartridge filters, because of their compact design, ease of use, and absolute microbial ratings, have become the major means to provide sterile gases in pharmaceutical processes.

Packed Towers

Description of Packed Towers

Packed towers used to filter fermentation air are comprised of beds which can be constructed of pads of paper, cotton wool, glass wool, or mineral slag wool. The diameter of the fibrous material is typically between 0.5 and 15 μm and the space between the fibers can be many times this range.

Figure 1 illustrates a simple design of a packed tower air filter (Stanbury and Whitaker, 1984). The filter consists of a steel container or housing filled with loose fibrous packing. The air inlet is on the bottom and the outlet is on the top of the filter. The packing is supported by a grid or perforated plate. When it is placed in the tower it is necessary to ensure that the appropriate packing density for the application is obtained. Proper packing must preclude movement of the fibers during use. A repositioning of fibers during use can lead to channeling of air, which will lead to an inefficient packed tower, since only a portion of the bed will be acting as a filter. Fiber movement can also lead to the dislodging of trapped microorganisms.

Once the filter has been correctly packed with fibrous material, a support grid or plate is fitted to the top of the bed to ensure that the bed remains compressed. After the first steam sterilization of a packed tower air filter, the packing will tend to settle further. It is recommended that additional fibers are added to the bed after the first steam sterilization to maintain the correct packing density.

Bonded fiber mats have been developed to use in place of loose fibers. When mats are used, it is necessary to have a good seal between the mat and the tower wall, so that channeling does not occur.

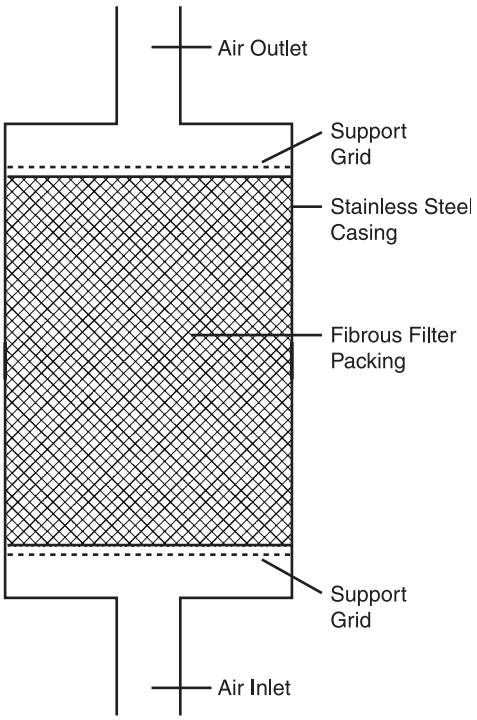


FIGURE 1 Packed air tower design.

Thin sheets of small diameter fibers can also be used in packed towers. The sheets are placed on top of each other with a mesh or grid in between each sheet for support. The edges of the sheets are sealed between flanges.

Sterilization of Packed Tower Air Filters

Prior to using a packed tower for the sterilization of fermentor inlet air, the filter itself must be sterilized. There are two techniques which can be employed: steam sterilization and dry heat sterilization (Richards, 1968).

Passing steam at a pressure of 15 psig through a packed tower for two hours should be adequate for sterilization. Because the presence of air in the packed tower during the sterilization can prevent complete sterilization of the packing, it is best to introduce the steam at the bottom and vent out the top of the housing.. A drain at the bottom of the packed tower is needed to purge the steam and to drain any residual condensate in the filter. It is necessary to remove condensate from the bed because wetted fibers are less efficient for particle and microorganism removal and may also decrease the retention efficiency of the packed tower well below its design value, particularly if channeling through the wetted media occurs.

Some fiber material, as well as material used to bond fibers, can be degraded by steam sterilization. An alternative to steam sterilization is dry heat sterilization, which will avoid the possibility of steam degradation and fiber wetting. This can be accomplished by using a heating device at the inlet of the tower and passing air at a temperature of 160–200˚C through the bed for two hours. During dry heat sterilization, the filter is isolated from the rest of the process. However care must be taken because not all materials can withstand the higher dry heat temperatures needed for sterilization.

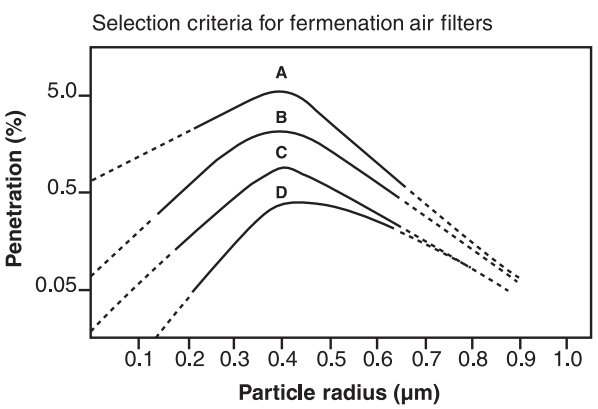
Operating Considerations for Air Sterilization by a Packed Tower

The microorganism retention efficiency of packed towers is dependant upon the velocity of the air within the tower. The relationship between air velocity and filtration efficiency has been determined experimentally in a number of studies (Fuchs, 1964). In the example shown in Figure 2 (Fuchs, 1964), the particle retention efficiency may change by a factor of 10 as a result of a relatively small change in the inlet air velocity. The results also show that the most difficult particles to remove are in the size range of small microorganisms. Therefore it is quite possible to encounter air flow conditions in a packed tower which can reduce the statistical probability for the complete retention of all microorganisms.

The hydrophilic nature of the fibrous material used in packed towers (e.g., glass wool) can contribute to a reduction of the microorganism removal efficiency of the packed tower. When water vapor enters the system with the air discharge of the compressor, the air and water vapor mixture is initially at an elevated temperature. As the gas stream is cooled, water droplets may condense, be collected by the fiber matrix and wet the hydrophilic glass fibers. Due to the loss of electrostatic effects, the wetted fibers are less efficient for microorganism removal and may decrease the retention efficiency of the packed tower well below its design value, particularly if channeling through the wetted media occurs. Also, organic components present in the compressor exhaust can provide a nutrient source for the retained microorganisms and increase the possibility of bacterial growth and eventual penetration through the depth filter medium. In addition, droplet formation can result in liquid being forced through the packed tower and contaminated droplets being re-entrained in the outlet air stream thereby contaminating the downstream system.

The pressure differential across the tower may also increase significantly with wetting, increasing the energy costs. A number of approaches have been tried to overcome the wetting problem, such as heat tracing to maintain an elevated temperature, these are expensive to operate and have not consistently resolved the problem.

Although it is obvious that operating deviations such as these do reduce the reliability of a packed tower for air sterilization, there is no quantitative procedure to determine if the filtration efficiency is adequate to assure a sterile inlet air condition. The



Mechanisms of aerosol filtration. Percent penetration of dioctyl phthalate particles through a resing impregnated glass fiber filter is plotted as a function of particle radius and incident air velocity (cm s¹). A.0.94: B.042: C.021: D.0.094

FIGURE 2 Particle retention efficiency.

lack of such a quantitative procedure adds an element of uncertainty which affects both the operation and maintenance decisions. Skilled operators are necessary to monitor the packed tower to ensure that it is packed and operating efficiently. The actual source of contamination of a production batch is often difficult to identify and the air filtration system is always suspect during a contamination outbreak. Without a technique for testing the efficiency of a packed tower, it is difficult to ascertain whether the packed tower is the cause of the contamination outbreak.

Design of Fibrous Air Filters

In order to model a fibrous air filter, several assumptions must be made. It is assumed that (1) once a particle is trapped by a fiber, it then will remain trapped; (2) at a particular depth across the filter, the particle concentration does not vary; and (3) the removal efficiency at a given depth is equivalent across the filter.

The following equation describes how the concentration of particles varies with the depth position in the filter (Richards, 1968):

$$\frac{dN}{dt} = -Kx \tag{1}$$

where N is the particle concentration, x is the depth, and K is a constant. Solving the equation between a depth of 0 and x and a particle concentration of N_0 particles entering the filter and particle concentration of N particles leaving the filter yields:

$$\ln\left(\frac{N}{N_0}\right) = -Kx \tag{2}$$

The relationship between depth and the logarithm of the ratio of particles removed to particles incident is known as the log-penetration relationship. (Richards, 1968). This relationship has been used in sizing depth filters.

The constant K in Equation (2) will vary with the type of packing and is dependant on linear velocity through the packed bed. If the relationship between the constant K and linear velocity through the bed is known, Equation (2) can be used to size a packed bed for a given log reduction of particles.

Another consideration for the design of a packed bed, is the pressure drop across the bed. Typically, the ΔP is linear with the linear air velocity for a given depth (Richards, 1968). The pressure drop across the packed tower can be dependant upon the type of medium, the packing density, the air density and the linear air velocity through the filter.

As an example of an equation for the pressure drop across the packed bed is given in Richards (1968):

$$\Delta P = \frac{2\rho v^2 \alpha x C}{\pi D_f} \tag{3}$$

where ΔP is the pressure drop, v is the linear air velocity, α is the ratio of filter density over fiber density, x is the filter bed depth, C is the drag coefficient, D_f is the fiber diameter, and ρ is the air density

At high air velocities, the above relationship indicates that the pressure drop is proportional to the square of the linear air velocity. At low linear air velocities (< 2–3 feet/sec), the relationship is linear.

Membrane Filter Cartridges

Unlike packed towers, cartridge filters are compact, easily handled, and can be used in housings or manufactured as a fully disposable capsule form. Their lack of depth is more than compensated for by their high surface area and tight particle capture specifications.

Membrane filters can have absolute or nominal ratings. A nominal rating is an arbitrary micron value assigned by the manufacturer, based upon the removal of some percentage of all particles of a given size or larger, and a comparison of nominally rated filters is very difficult. Nominal ratings can also be misleading, because the filter is not absolute at the pore size rating and can pass particles larger than the rating indicates.

An absolute pore rating can be defined as the diameter of the largest spherical particle that will pass through a filter under specified conditions. It is an indication of the largest available pore opening in the filter elements. The ratings are determined under carefully controlled conditions using industry accepted reference standards. These include silica suspensions, latex beads, or microbial organisms.

Membrane filter cartridges are available as either prefilter (particulate contaminant rating) or sterilizing filter (bacterial contaminant rating) configurations. Prefilters in air service can be used for particulate removal or aerosol removal. Prefilters are positioned upstream of the final (sterilizing) filters and are designed to protect the final filter from premature plugging, thereby prolonging significantly the life of the final filter. The following are brief descriptions of the cartridge filters (sterilizing and prefilter types) that can be used for air filtration.

Prefilters

For some applications it is essential to use a pre-filter to remove particulate material or liquid aerosols, from the incoming steam, compressed gas or piping, present in the gas stream. This prefilter reduces or eliminate particulate contamination within the system and can protect and extend the life of the sterilizing grade filter. Typically, the use of a pre-filter will significantly enhance the overall filtration economics.

Particulate Removal

Membrane filters composed of materials such as, for example, polypropylene or cellulose can effectively be used to remove particulate material from an air stream. The filter micron ratings range from the order of 1 to the order of 100 μm. The appropriate filter can be selected for the desired level of particulate contaminant removal. The following are descriptions of examples of filters that can be used as prefilters in pharmaceutical air filtration.

Porous Stainless Steel Filters

Porous stainless steel medium is made by sintering very fine particles of stainless steel or other high alloy powder to form a controlled pore size metal medium. When used as a filter medium, porous stainless steel can be formed as a flat sheet, or preferably as a seamless cylinder. This special manufacturing process produces a high dirt capacity medium which is temperature and corrosion resistant. The recommended alloy is type 316LB, which has a higher silicon content than type 316L and provides a stronger, more ductile product with better flow properties.

Standard porous stainless steel filters are 2 3/8 inches O.D., with type 304 or 316 stainless steel flat blind end cap at one end and 1 or 1.5 inch NPT connection at the other, or industrial style flat gasket open end caps welded at each end. Porous stainless steel filter cartridges are chemically or mechanically cleanable, offering economy of reuse. For gas and steam applications, elements typically have absolute ratings of 0.4–11 μm. They are also very useful in sparging applications.

Cellulose Pleated Filter Cartridges

Pleated cellulose filter cartridges are applicable as prefilters for inlet air for fermentors and bioreactors. These filter cartridges are constructed of pure cellulose medium, without resin binders, which is pleated into a high area cylinder.

Cellulose media cartridges are assembled with hardware components consisting of a perforated inner support core, an outer support cage, and end caps melt sealed to imbed the medium in the plastic. All hardware components are typically of polypropylene.

Polypropylene Pleated Filter Cartridges

Polypropylene pleated filters are applicable as prefilters for prefiltration of inlet and exhaust gases. These process filter cartridges are constructed using non-migrating continuous strands of non-woven polypropylene filaments. The filter should have an absolute rating for reliable selection and performance. The thin sheet of polypropylene media is pleated and formed into a cylinder with a longitudinal side seal of melt seal polypropylene. The cylinder is then melt sealed to molded polypropylene end caps to ensure no fluid bypass. Polypropylene hardware components consisting of an inner support core and an external protective outer cage are incorporated.

Liquid Aerosol Removal

A coalescer can be used for the removal of liquid aerosols containing water or oil droplets. This is desirable in compressed air systems and in fermentor vent applications as a pre-filtration for a sterilizing air filter, because the liquid aerosol could prevent the flow of air through the filter.

Coalescers operate efficiently if they are able to separate the liquid and the gas in the liquid aerosol. The three basic steps that are required are:

- aerosol capture,
- unloading or draining of the liquid, and
- separation of the liquid and gas.

Sizing of liquid/air coalescers is critical if they are to perform their function. They must be matched to the air flow and that flow must be below the rate at which the coalesced droplets will be swept off of the medium and re-entrained into the air exiting the unit.

Figure 3 is an illustration of a liquid-gas coalescer. The coalescer in Figure 7 has a gravity separator, which allows for the removal of large liquid aerosols (typically > 300 μm). The coalescer flow direction is in to out to prevent re-entrainment. The liquid is captured through the coalescence of fine aerosols (0.1–300 μm) to large droplets (1–2mm). The coalescence of the droplets is illustrated in Figure 4. The large droplets flow downward from a drainage layer. The separated liquid is then drained, usually automatically, from the system. The aerosol-free air leaves the system from the top of the assembly.

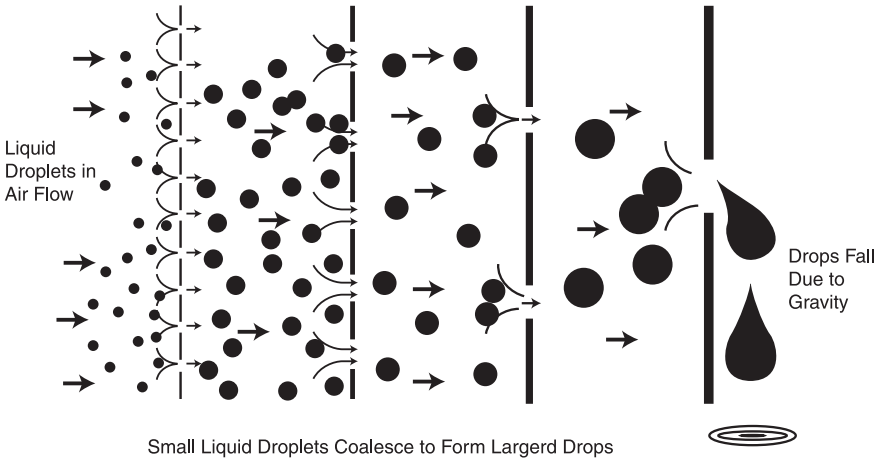


FIGURE 3 Coalescence of droplets.

Filter Housings

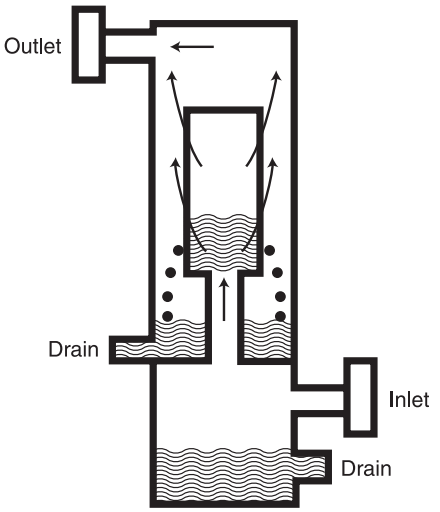
It is also necessary to consider the performance requirements of replaceable cartridge type sanitary, air/gas service filter housings. Housing size should be adequate for the flow and differential pressure requirements. Filter housings for pharmaceutical applications are typically constructed of stainless steel (e.g., 304, 316, 316L, etc.) or carbon steel, with 316 series stainless steel internal hardware and cartridge seating surfaces. Internal hardware includes tube sheet adapters, tie rods, and seal nuts. Housing closures should utilize quick release mechanisms such as V-band clamps or fast-action swing bolts to facilitate filter change-outs.

Design operating pressure of all filter housings should be specified as maximum psig, and, where needed, rated for full vacuum service. Design maximum operating temperature of the housing should also be specified. The housings or pressure vessels which are within the scope of the ASME Boiler and Pressure Vessel Code, Section VIII, Division 1, should be designed and U stamped per the code. TIG weld construction should be used in sanitary style housings to minimize weld porosity and insure high quality, clean joints, with all internal welds ground smooth and flush. All weld procedures and welders should be qualified to ASME/BPVC Section IX. In addition, the finished surface should be polished to the level demanded by the end use application up to and including electropolish.

Housings should be capable of in situ steam sterilization in accordance with the manufacturer's recommended procedures and housing or system design should provide for condensate drainage. Gasket material and O-ring elastomers must also be capable of withstanding repeated steam sterilization cycles, along with being compatible with process fluids.

Industrial style housings provide cartridge mounting on a tie rod and sealing to the tie rod assembly by use of a seal nut at the top of the assembly. Tube sheet adapters should be seal welded to the tube sheet to prevent fluid bypass. Filter cartridges are thereby sealed in the housing independent of any cover assembly, ensuring positive sealing and no fluid bypass. Filter cartridges should be seated on the tube sheet adaptor assemblies above the tube sheet to ensure complete drainage of non-filtered fluid or condensate prior to cartridge replacement. This prevents potential contamination of downstream surfaces during change-out of filter elements.

Air Filtration Applications



Gravity Separator: Removal of large liquid aerosols (>300 µm).
Coalescer: Flow DirectionL In-to-out; -prevents reintrainment.
Capture: Coalescence of fine aerosol liquids (0.1-300 µm) to large droplets (1-2 mm).
Drainage: Downward flow of liquids from coalescer drainage layer.
Separation: Removal of liquids from housing.

FIGURE 4 Liquid-gas coalescer.

Stainless Steel Housings for Air/Gas Service for sterilization applications

Air/gas service housings should be designed to maximize air/gas flow with minimal pressure drops. The preferred material of construction is again type 316L stainless steel where in contact with the fluid stream. Other stainless steel grades may be used for external hardware. Internal surface should be mechanically polished to a uniform "Scotch Brite" finish, while external surfaces can be mechanically polished to a high quality finish. After polishing, the entire housing should be passivated.

Inlet and outlet fittings for air/gas service housings should either be 150 LB ANSI R.F. SO Flange or SCH 10S Butt Weld, with appropriate wall thickness as per pipe diameter specifications. Vent and drain ports should be stainless steel fittings with appropriate thread diameters for the housing size.

Design of Sterilizing grade Membrane Filters for Air Service

Membrane filters used for fermentor and bioreactor sterile inlet air and exhaust gas vents, sterile pressure gas, sterile nitrogen blankets, storage tank sterile vents, formulation tank sterile vents, lyophilizers and sterile air for aseptic packaging, usually contain a membrane made of hydrophobic materials such as polyethersulphone (PES), polyvinylidenefluoride (PVDF), or polytetrafluoroethylene (PTFE).

It should be noted that prior to the introduction of hydrophobic membrane filters, hydrophilic (e.g., nylon, modified PVDF and PES) were used for air filtration applications in the pharmaceutical industry. Today, while hydrophobic membrane filters are employed for most air applications, there do exist applications in which hydrophilic

membrane filters are used. Hydrophilic membranes can be used for dry air and gas filtration in applications in which the moisture is minimal (no free liquid.).

Hydrophobic membrane filters are preferred in sterile gas filtration applications because hydrophobic filters do not wet with water. When a hydrophilic filter membrane is wetted with water, it will not pass air until the water-wet bubble point of the filter is exceeded. This water-wet bubble point can be greater than 50 psi. The inherent hydrophobicity of membrane filters used for air sterilization allows sterilizing grade filters to be able to remove bacteria and viruses completely from air, even when exposed to moisture (Bruno and Szabo, 1983).

The hydrophobic membrane filter material is pleated together with a layer of material (typically non-woven polypropylene) on the upstream and downstream side of the membrane. These layers provide mechanical support to the membrane and proper drainage of the fluid. The pleated membrane pack is formed into a cylinder and a longitudinal, homogeneous melt side seal is made.

A rigid, perforated inner core is present to provide support against operating pressure. An outer cage placed on the upstream side of the membrane filter pack is provided for additional support and protection during handling. The cage provides retention of structural integrity against accidental reverse pressure. Polypropylene and PTFE are examples of materials that can be used for the cage, core and support material in a hydrophobic membrane filter.

End caps are attached by melt sealing to imbed the membrane pack in the plastic. Multi-length elements can be welded together end to end to produce larger units. Membrane filters are available in a variety of shapes and sizes. The most typical configuration for pharmaceutical air applications are 10 inch elements. A membrane filter is illustrated in Figure 5.

Membrane filter elements may be integrity tested in order to assure that they are integral and will provide air that is sterile. Using an integrity test that has been correlated with bacteria removal efficiency.

Sterilization of Membrane Filters

During use hydrophobic membrane process filter cartridges may be subjected to multiple sterilization cycles and, therefore, must be designed to be repeatedly steam sterilized in either direction of flow or repeatedly autoclaved. The vendor can provide guidelines on filter cartridge sterilization and use limitations (time & temperature). Typically these filters are capable of withstanding multiple autoclaving or in-situ steam sterilizations of over 100 cumulative hours at up to 140˚C.

Because the filters are hydrophobic, drying time is usually not required prior to starting the gas flow in the system. It is necessary, for membrane filters, as well as for packed towers, to drain the entrained condensate on the inlet side of the filter. It is essential that the proper steam condensate traps be in the system and that the steam be maintained as dry as possible to prevent condensate filling the housings and blocking air flow.

QUALIFICATION TESTS FOR MEMBRANE FILTERS

Sterilizing Grade Membrane Filters

Organism Retention Tests

Microorganism retention tests can be conducted to verify that membrane filters produce sterile air. Liquid challenge tests with *Brevundimonas diminuta* (ATCC 19146),

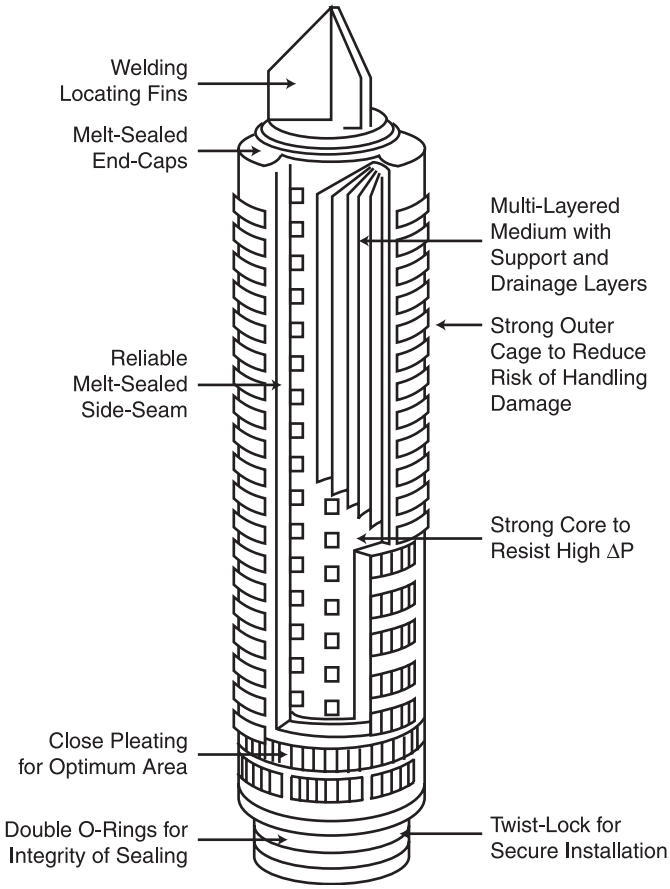


FIGURE 5 Membrane filter (Haughney, 1995).

measuring 0.3×0.6–$0.8\,\mu m$, is a standard challenge test for the validation of sterilizing grade filters ($0.2\,\mu m$) in the pharmaceutical industry. Aerosol challenge tests with *Brevundimonas diminuta* should approximate process air flow conditions. Aerosol challenges with T_1 bacteriophage ($0.05 \times 0.1\,\mu m$), or *PP7* (25 nm) can provide a test of a filter's retention efficiency of extremely small organisms in air service

The retention efficiency of a given filter is less when a liquid challenge is used instead of an aerosol challenge. Thus, a liquid challenge test is a more stringent test of a filter's retention capability. A liquid challenge test can also provide retention information for process conditions such as extreme moisture after sterilization or air entrained with water drops. An example of the technique used to perform a liquid bacterial challenge on sterilizing grade membrane cartridges is found in ASTM Standard Method F838-05

The industry standard liquid test for sterilizing grade filters involves challenging a test filter with a known quantity of *Brevundimonas diminuta*, no less than 1×10^7 organisms per cm^2 of filter area. The challenge sample is passed through the filter suspended in sterile water at a defined flow rate and time. All of the effluent from the test filter is passed through an analysis membrane. The analysis membranes are removed and placed on an appropriate agar growth medium at 32°C for 48 h. After incubation, the plate

is examined for the presence or absence of microbial colonies. Figure 6 illustrates a liquid bacterial challenge test stand.

The aerosol challenge test system can consist of a nebulizer loaded with the challenge microorganism suspension, a separate line for dry air make-up, and split stream impingers to sample the aerosol challenge with and without the test filter. A schematic of a test stand that can be used for the aerosol challenge procedure described is given in Figure 7. There are a multitude of publications describing aerosol tests in detail (Meltzer, 1987; Pall, 1994; PDA TR 40, 2005).

During the aerosol challenge, an aerosol is generated with a nebulizer. The aerosol is introduced into the test filter at a given flow rate. The filter effluent is collected in dual liquid impingers. Controls are performed simultaneously via a split stream by using a two channel timer to direct air flow, on an alternating basis, from the test side filter impingers to the unfiltered control side impingers for recovery.

The impingers contain sterile buffer and after the challenge is completed, the buffer can be analyzed for the test organism. If *B. diminuta* is the test organism, then the buffer is analyzed by putting the buffer solutions through an analysis membrane and placing the membrane on Mueller Hinton Agar for 48 h, before titering. If a bacteriophage is the test organism, then samples of the buffer are diluted with Nutrient Broth and mixed with liquid Nutrient Agar (0.7% agar concentration; 48˚C) and *E. coli* in the log phase of growth. After mixing all three components, the mix is poured over Nutrient Agar plates and incubated for at 37˚C, so that the plaques can be counted.

Test Certificate

To verify and document the Quality Assurance testing of filters optimized for pharmaceutical use, the vendor shall provide a Certificate of Conformance' with sterilizing grade filters.

Materials of Construction. All materials used in the manufacture of filters optimized for pharmaceutical use shall be traceable. All filter components shall be made from materials listed for food contact usage per Title 21 of the U.S. Code of Federal

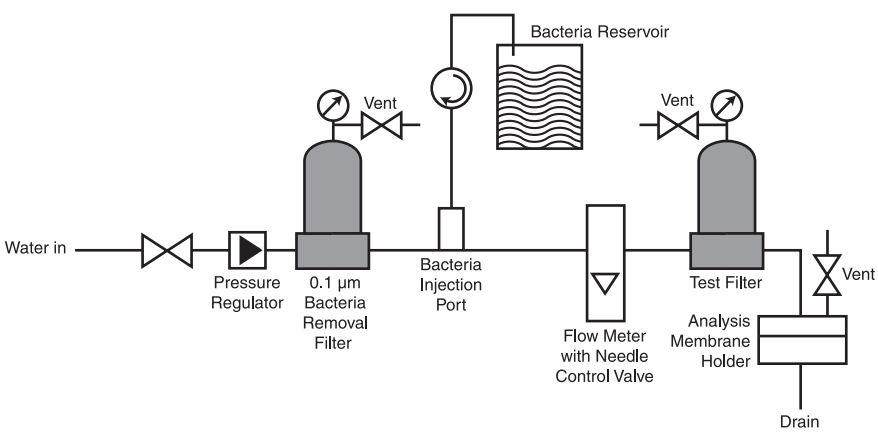


FIGURE 6 Liquid bacterial challenge test stand (Haughney, 1995).

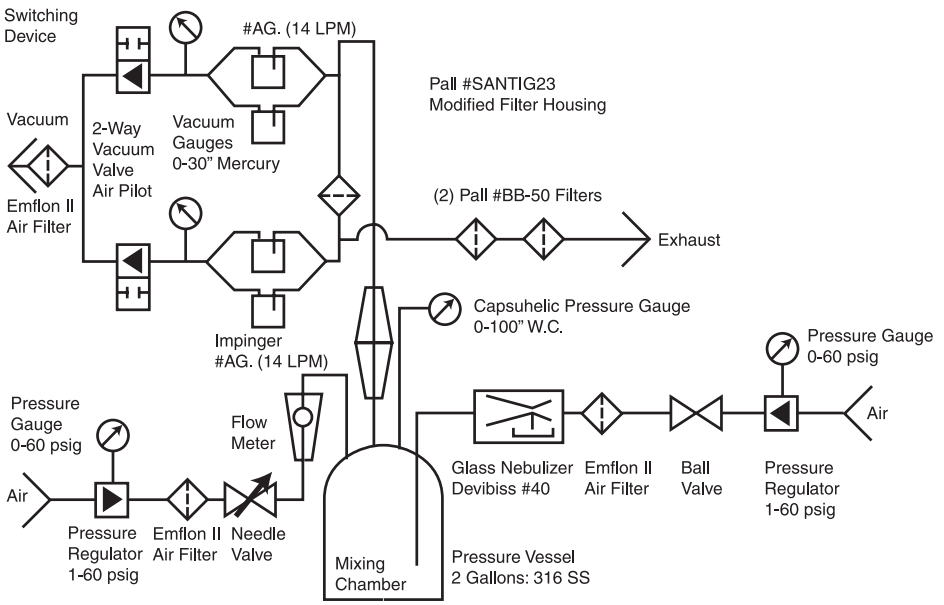


FIGURE 7 Aerosol challenge test stand. *Source*: Courtesy of Pall Corporation.

Regulations (CFR), parts 170–199. These materials shall also meet the specifications for biological tests listed in the latest revision of the United States Pharmacopeia (USP) for Class VI plastics at 121˚C.

Element Integrity. All sterilizing grade filter elements shall be 100% integrity tested non-destructively (e.g., forward flow) to validate the absolute rating and integrity of the filter element. The integrity test procedure shall be correlated to a bacterial challenge test.

Effluent Quality. Filter samples from each manufacturing lot shall undergo the following tests to assure the highest level of effluent quality.

Cleanliness	Shall meet the USP limits under Particulate Matter in Injections with effluent counts determined microscopically. Counts shall serve to document conformance with the requirements for a non-fiber releasing filter per 21 CFR 211.72 and 210.3 (b) (6).
Oxidizables	Shall meet the USP requirements under Purified Water after flushing.
pH	Shall meet the USP requirements under Purified Water with effluent and influent samples, taken after the flush for oxidizable substances.
Pyrogens	Shall meet the USP requirements under the Bacterial Endotoxins Test with an aliquot from a soak solution containing less than 1.0 EU/ml, as determined using the limulus amebocyte lysate test.

DOP Test

The DOP (dioctylphthalate) aerosol test has seen extensive use in measuring the efficiency of HEPA and ULPA filters used to provide air to clean rooms, and for similar applications. The commercially available equipment operates at 4 CFM, and cannot detect fractional penetration by 0.3 μm droplets of 10^{-6} (0.0001%). Using microbiological terminology, the method, at 4 CFM, is able to measure titre reduction (defined as the ratio

of upstream to downstream colony forming units) of 10^6. It cannot, for example, distinguish between two filters having respective titre reductions of 10^8 and 10^6, nor can it detect a minor defect that could compromise sterility of the gas filtered

A typical air flow for a fermentor air filter, such as a sterilizing grade 0.2 μm rated membrane filter, is about 50 to 100 SCFM per 10 inch module. To test a single module at 50 SCFM, for example, the DOP method requires a 12.5-fold dilution with air, reducing the sensitivity to a titre reduction of 8×10^4. To test in situ at a 1000 CFM rate reduces sensitivity further to 4×10^3. A titre reduction of 10^4 corresponds to a removal efficiency of 99.99%. This means that this test is basically a qualitative check for filter damage when used with all but the smallest housings.

As an example, the bacteria content for the compressed air incident on a filter could range from 10^1 to 10^4 per cubic foot, depending on the cleanliness of the system. Assuming a level of 10^2 organisms per cubic foot, a single 10 inch module run at 50 SCFM for 180 h would have approximately 5.4×10^7 bacteria incident on it. A filter able to meet the DOP test sensitivity of 8×10^4(~99.998%) could theoretically pass 6.8×10^2 bacteria during the 180-h period (one every 15 min).

By contrast, the bacteria challenge test and its relationship to the industry standard integrity tests, the Forward (Diffusive) Flow, Water Intrusion, and Bubble Point, provide reliable and convenient means to test the removal capability of a sterilizing grade filter. It is the method upon which most filter manufacturers' validation data is based. This test represents a worst case condition.

As with the liquid challenge test, for a filter cartridge to pass an aerosol challenge, all incident organisms must be retained. The test reliability is such that a single organism downstream can be detected. Thus, using the aerosol challenge tests a titre reduction of $> 10^{10}$ for *Brevundimonas (Pseudomonas) diminuta* (size: 0.3×0.8 μm) and a titre reduction of $> 10^8$ for T_1 bacteriophage (size: 0.05×0.1 μm) can be demonstrated. A DOP test, under the same conditions, would only be able to demonstrate a titre reduction of about 8×10^4 (efficiency of 99.998%), due to the limited sensitivity of the test.

Furthermore, frequent DOP testing of a membrane filter cartridge may reduce the service life of the filter. An accumulation of the DOP (or surrogate) oil droplets generated during the test can be deposited on the filter, accumulate as an oil film, and block the pores of the media.

Integrity Tests for Membrane Filter Cartridges

For sterilization of fermentor air, it is necessary to achieve the highest possible assurance of filter integrity and removal efficiency. The installation of integrity-testable filters and the performance of routine integrity testing by the user is essential to demonstrate that the system is performing to specification. Tests which qualify the retention characteristics of a membrane filter can be defined as destructive or non-destructive tests.

Destructive tests are performed using an appropriate contaminant to meet a specific claim for retention of the contaminant. The test procedure must be sensitive enough to detect the passage of contaminants of interest. For sterilizing grade 0.2 μm membrane filters, the industry standard test organism (i.e., contaminant) is *Brevundimonas diminuta* (ATCC 19146). The organism and minimum challenge level (10^7 CFU/cm^2 filter area) are specified in the ASTM standard F383-05 (2006) and referenced in the FDA Guideline on sterile drugs produced by aseptic processing, (ASTM, 1983; and FDA, 1987). The current 2004 FDA Guideline specifies that a sterilizing grade filter is one that removes all microorganisms present in the product being processed.

Because most filter users would not want to perform a destructive test in a process environment, non-destructive tests [Forward (Diffusive) Flow, Bubble Point, Water Intrusion] related to the retention results of the destructive test are used instead. From the relationship developed between a non-destructive and a destructive test, membrane filter performance can be safely and conveniently verified in the production environment. The relationship between a non-destructive integrity test and the assurance of bacterial retention constitutes a filter validation study, and is extremely important for microbial retentive filters used in critical fluid processes.

Tests for Prefilters

Particulate Tests

Many filter manufacturers use a nominal micron rating for removal efficiency. This is defined by the American National Standards Institute (ANSI) as an "arbitrary micrometer value indicated by the filter manufacturer. Due to lack of reproducibility this rating is deprecated."

An alternative method for rating filters is the Oklahoma State University (OSU) F-2 Test. This rating method (ISO 4572, ANSI B93.31) provides a standardized absolute rating and has received wide acceptance for use on lubricating and hydraulic fluids and has been adapted for use in water based tests.

The test is based on continuous on-line particle counts of different particle sizes, both in the influent and the effluent. The Beta ratio at a specific particle size is defined as β_X: the number of particles of a given size (X) and larger in the influent, divided by the number of particles of the same size (X) and larger in the effluent, where X is the particle size in microns. The percent removal efficiency can be calculated from the Beta value. The percent removal efficiency is $[(\beta_X - 1)/\beta_X]$ 100. (Uberoi, 1992)

As an example, Pre-filters are given a micron rating that corresponds to a 100% removal efficiency or the value in microns at which the OSU F-2 Test gives a Beta value of > 5000.

Aerosol Removal Tests

A coalescer effects a separation of liquid and gas by first capturing the aerosol, then unloading or draining the liquid, and finally separating the liquid and gas Coalescer performance can be degraded by re-entrainment of the discontinuous (or liquid) phase due to poor drainage. It is preferable, therefore, to use a coalescer performance test that employs an aerosol and parameters representative of actual systems and considers the three factors (aerosol capture, medium drainage, and downstream separation) of importance for proper operation. (An example of such a procedure is found in Williamson et al., 1988)

AIR FILTRATION APPLICATIONS

During the processing of a pharmaceutical product, the following are some of the basic processing categories:

- fermentation inlet gas,
- fermentation off-gas,
- product contact gases,

- vent filters – compendial water (including hot and ozonated),
- vent filters – product holding tanks.

For each of the above steps, sterilizing grade 0.2 μm hydrophobic membrane filters can be required for processing air (or gas) streams.

For example, sterilizing grade 0.2 μm hydrophobic membrane filters are used during:

1. fermentation—for the sterilization of fermentation inlet air, as well as, for the filtration of fermentation exhaust gas,
2. downstream processing—as sterile tank vents, and
3. final purification—for vacuum breaking in processes such as lyophilizers and autoclaves.

As discussed above, hydrophobic membrane filters are desired in sterile gas filtration applications since hydrophobic filters do not spontaneously wet with water.

The Parenteral Drug Association (PDA) Gas filtration Committee has in PDA document TR40 has defined the desirable characteristics of these filters for these applications and the guidelines for use of hydrophobic filters in eight applications (A–H) as follows:

"Most applications for hydrophobic membrane filters can be satisfied with a filter that meets as many of the following ideal characteristics as possible:

- The filter must retain microorganisms, even under adverse conditions such as high humidity.
- The filter should have high thermal and mechanical resistance, sufficient to endure long-term applications under demanding use conditions.
- The filter should withstand multiple steam sterilization cycles.
- The filter should allow high gas flow rates at low differential pressures.
- The membrane should be hydrophobic to resist blockage by condensate.
- The filter construction should be optimized for long, dependable service life.
- The filter must not release fibers.
- The filter must be integrity testable with a test correlated to removal efficiency.
- The filter should be easy to install and maintain.
- The filter's materials of construction should be compatible with the proposed application (e.g., oxygen service)

The relative importance or need for such properties can best be illustrated by a few sample applications.

Product Contact Gases

The broadest, most critical use of sterilizing grade hydrophobic membrane filters is for gases that are in direct contact with pharmaceutical products. For example, nitrogen gas is widely used to blanket oxygen sensitive solutions to reduce degradation. Any gas that comes in contact with solutions should be sterile to maintain low bioburden in terminally sterilized products or to maintain sterility in aseptically filled products. This includes process gases used in tanks or headspace gases used to flush product vials and ampoules.

Due to the critical nature of these applications, hydrophobic membrane filters that are validated to a rigorous liquid-based microbial retention challenge are recommended. In many critical applications, redundant filters in series are frequently employed, but not

required. Filters must be routinely integrity tested in use to assure their efficacy. Membrane materials should be chosen to reflect the conditions of use, especially if filters units are steamed- or sterilized-in-place.

Fermentor Inlet Air

The volume of air required to maintain the fermentation process depends on the process and the volume of the culture, and filtration systems should be sized accordingly. In large fermentor applications, the air supply may be millions of cubic meters per year and require large filter assemblies. The air supply needs to be reliable to provide proper oxygenation of the culture and sterile in order to avoid costly contamination problems in the process. Filters used in fermentation processes should meet high microbial retention standards and provide high flow rates at a relatively low pressure drop (1–5 psig). Membrane materials for such applications should be hydrophobic, of high void volume, yet show reliable microbial retention capability. Construction of the filter cartridges is optimized to avoid water blockage. The elements also require a high thermal and mechanical stability, because for the process to be economical, they have to withstand many sterilization cycles at elevated temperatures.

Fermentor Off-Gas

Membrane filters are employed increasingly for fermentor off-gas applications. The challenges in this application are the high moisture content and the high level of microbial contamination of the fermentor exhaust gases. As the gas stream cools, condensation occurs. This, in turn, can result in an undesirable increase of the head pressure within the fermentor. Water blockage can be avoided by choosing the proper design, protecting the final filter with coalescing pre-filters and heat tracing the filter housings to avoid condensation. Off-gas systems should be designed to prevent condensate and coalesced aerosols from reaching the filter. This is often accomplished by having the off-gas condensate drain back into the fermentor.

Also, there is a potential for foam to be carried over into the off-gas, which can lead to blockage of the filter. Therefore, systems should be designed and operated to avoid foaming. Foaming is typically reduced with addition of anti-foam agents or modification of the fermentation media. In difficult processes, it may be necessary to install a mechanical separator to eliminate foam and the potential for filter blockage.

Vent Filters on Compendial Water and Product Holding Tanks

When liquid is added to or drawn from a tank, an equivalent volume of air is displaced from or into the tank. To avoid bacterial contamination of the contents in critical applications, the air has to be filtered through a sterilizing grade vent filter. The same is true when a holding or transport tank is steam sterilized, because the air that enters the tank at the end of the sterilization cycle has to be sterile. In addition to the rigor of the steam cycle, another challenge presented by this application is blockage of flow due to entrapment of moisture within the membrane.

The need to avoid blockage of flow through the vent filter is particularly important at the end of a steaming cycle. As the tank cools, condensation of steam creates a vacuum that can be estimated from the ideal gas law or steam tables (Meltzer 1987b). At 100˚C, for instance, each liter of steam that condenses will occupy only about 0.6 mL, an almost

1700-fold decrease in volume. Because the bulk of the condensation will take place rapidly, the vent filter should be properly sized to deliver the equivalent of the tank volume of air in a small period of time. If no appropriate measures are taken to prevent the disruption of air flow through the vent filter, the resulting vacuum in the tank may damage the tank. The issue is less problematic in tanks that are vacuum rated, a feature that makes them considerably more expensive.

Other design features can also prevent tank implosion. For instance, the vent filter can be connected to a source of compressed air, at a pressure high enough to displace the moisture lodged within the pore structure. Preventive measures such as heat traced housings should be seriously considered. Special care needs to be exercised in the sizing of the filter to avoid the problems associated with blockage in this application. It is also prudent to fit sealed tanks with a suitable rupture/implosion disc. However, reliance on this feature risks product loss and implies a significant amount of downtime to replace the disc, as well as repeating the cleaning and steaming process.

Lyophilizer and Autoclave Vacuum Break

The air (gas) that enters the chamber of a lyophilizer will come into direct contact with the sterile product. Likewise, the air that enters an autoclave will come in contact with sterile commodities or equipment. Hence, the gas supplied to reduce/break the vacuum at the end of the lyophilization/autoclave cycle must be sterile in these cases. Disruption of air flow due to condensation can adversely impact the operation, and appropriate measures to prevent this should be taken. The filter element in such applications needs to be sterilized, most often by steaming in place. The filter manufacturer's recommendations for steaming or sterilization should be adhered to, particularly if steaming in the reverse direction is required. Because the filter may be subjected to repeated steaming or sterilization cycles in such applications, it should be durable, and should be integrity tested on a regular basis to assure the expected microbial retention level. Ease of integrity testing, placement of the filter, and easy access to the filter are critical in this application.

Gas Used for Drying and Transfer/Fill Line

Some components (such as rubber stoppers and large equipment such as holding tanks) are typically rinsed in WFI and dried after steaming. Drying is especially critical if they are to be used in oil-based sterile product formulations. Often, compressed air is used to accelerate the drying process.

In addition, in many processes, the sterile bulk product must be transferred from the sterile holding tank to the filling line. This is often accomplished by pressurizing the head space in the holding tank with a suitable gas.

The gas in such critical applications must be sterile and free of particles, and a suitable filter must be chosen. Filters in such applications should be routinely sterilized and integrity tested to assure the expected microbial retention capability.

Blow-Fill-Seal Equipment

Large amounts of sterile compressed air are needed to run blow-fill-seal operations. Often, the equipment is fitted with several different air filtration systems in order to provide sterile air to individual process steps, such as in molding the primary container or

shielding critical portions of the machine to prevent the ingress of environmental air containing bacteria and particulate matter. The filtered air contacts critical surfaces as well as the product during the filling step, thus, a high level of bacteria retention must be assured through proper filter selection and validation. Form-fill-seal operations are typically run for extended times, thus the filters used must be durable and reliable. The filters must be routinely sterilized and integrity tested to assure the expected retention performance.

Environmental Air in Isolators

Isolator technology has been gaining popularity over the past few decades for critical applications, such as sterility testing, aseptic filling, weighing and handling of sterile and even non-sterile potent compounds. Depending on the application, isolators can be run at positive or negative pressure relative to the surrounding environment. Whichever the mode of operation, filtration of make-up and exhaust air plays an important role. Hydrophobic membrane filters can be used as an alternative to conventional depth filters, such as HEPA filters to accomplish the air exchange between isolators and the surrounding environment. The more demanding the operation, whether it be retention of toxic powders from the exhaust or the admission of sterile air, the more demanding the retention validation and integrity test program that should be implemented."

Fermentation Air Filtration Applications

During a fermentation process a specific cell (yeast, bacteria or mammalian) is grown to provide a desired product. Products can include cells, antibiotics, amino acids, or recombinant proteins. There can be a variety of sizes for the fermentor, ranging from very small (100 L or less) cell culture reactor to very large scale antibiotic production (100,000 L). In these applications there is often the need to maintain sterility in both liquid and gas (air or nitrogen) feeds to support growth of the desired cells.

Air filtration applications for fermentation are shown in Table 1 and illustrated in Figure 8. Those applications specific to fermentation are described below, while filtration of utilities used in fermentation such as steam, air, and water are discussed in the Utilities section of this chapter.

Prefiltration of Fermentation Air

Compressors are used to generate air flow for the manufacturing facility. There are two types of compressors, oil free and oil lubricated. In older facilities where oil lubricated air compressors are commonly used, prefiltration of inlet air is necessary for removal of oil droplets. A coalescing filter, can provide greater than 99.9% removal of oil and water droplets in the 0.01–0.5 μm range and larger. This also acts as an excellent prefilter for the hydrophobic membrane pleated filters that are commonly used for sterilizing the inlet air to the fermentor.

For oil free compressors, a prefilter acts to remove dirt in the air system, extending the service life of the final filter. For use with fermentation air, a cellulose pleated filter with an absolute rating of 8.0 μm is normally the filter of choice. Alternately, polypropylene (2.5-μm-rated) pleated filters also serve as excellent prefilters for this application.

TABLE 1 Filter Recommendations for Fermentation Air Applications

Application	Filter type	Micron rating	Typical gas flow rate per 10 inch module SCFM
Prefiltration of air to sterilizing filter for particulate removal	Cellulose pleated	8.0	75
Prefiltration of air to sterilizing filter for oil droplet removal (coalescer)	Polypropylene pleated	2.5	200–400
	Coalescer	0.3	
Sterile filtration of air for fermentors and bioreactors	Hydrophobic membrane	0.2	75–100
Sparging	Porous stainless steel	3.0	
Prefiltration of exhaust gas from fermentors and bioreactors	Polypropylene pleated	1.2	40
Sterile filtration of exhaust gas from fermentors and bioreactors	Hydrophobic membrane	0.2	40
Tank venting	Hydrophobic membrane	0.2	75–100
Steam for cleaning and sterilizing	Porous stainless steel	18	30 psig saturated steam (130 lb/h)

Sterile Air Filtration for Pilot and Production Fermentors and Feed Tanks

One of the largest applications for sterile air filtration is the air used for an aerobic bioreactor or fermentor during a typical production cycle. Typically, one volume of air per volume of broth per minute is used. Thus, for a 10,000 L fermentor on line for 48 h a total of 1×10^6 cubic feet (2.8×10^4 Ncbm) of air requires sterilization.

The contaminants present in compressed air can include the following: dust, lubricating oil, hydrocarbons, water, rust, and microorganisms including molds, bacteria, and viruses. Microorganisms in air are often associated with carrier particles, such as dust. Water and oil can be present as bulk liquid, vapor or an aerosol. The air distribution system can give rise to contaminants such as rust and water. The concentration and size distribution of particles in compressed air are variable. The size range is generally between 0.001 and 30 μm, with a concentration between 10^{-2} and 10^{-4} g/m^3 (Richards, 1968; Bruckshaw, 1973; Jornitz, 1998).

Bacteria and bacteriophage, when present in air feeds, can enter fermentation tanks or bioreactors and contaminate the product. Bacteriophage or other viruses can destroy the producing cells and reduce yields.

The process requirements to supply this sterile air are quite restrictive. The air sterilization process must: (1) process a large volume of compressed air, (2) provide a high degree of reliability, and (3) operate economically. Several methods have been considered for the sterilization of fermentor inlet air. These include: filtration, heat, irradiation, washing with sterilizing chemicals, and electrostatic precipitation. Washing and electrostatic precipitation are not effective for the removal of microorganisms. Heat and irradiation are not economical. Filtration is the only technique that meets all the requirements for sterilizing bioreactor and fermentor inlet air.

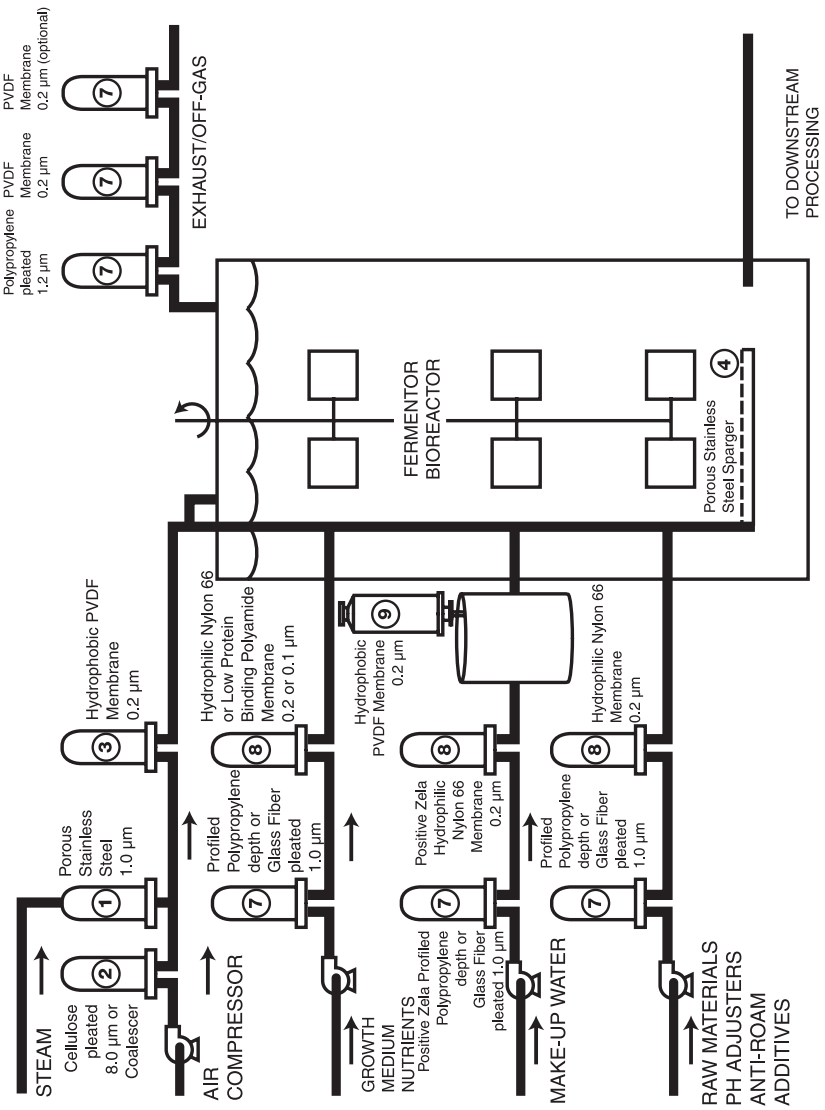

STEAM
Cellulose pleated 8.0 µm or Coalescer
Porous Stainless Steel 1.0 µm
Hydrophobic PVDF Membrane 0.2 µm
AIR COMPRESSOR
Profiled Polypropylene depth or Glass Fiber pleated 1.0 µm
Hydrophilic Nylon 66 or Low Protein Binding Polyamide Membrane 0.2 or 0.1 µm
GROWTH MEDIUM NUTRIENTS
Positive Zeta Profiled Polypropylene depth or Glass Fiber pleated 1.0 µm
Hydrophobic PVDF Membrane 0.2 µm
Positive Zeta Hydrophilic Nylon 66 Membrane 0.2 µm
MAKE-UP WATER
Profiled Polypropylene depth or Glass Fiber pleated 1.0 µm
Hydrophilic Nylon 66 Membrane 0.2 µm
RAW MATERIALS
PH ADJUSTERS
ANTI-ROAM
ADDITIVES
FERMENTOR BIOREACTOR
Porous Stainless Steel Sparger
Profiled Polypropylene pleated 1.2 µm
Hydrophobic PVDF Membrane 0.2 µm
Hydrophobic PVDF Membrane 0.2 µm (optional)
EXHAUST/OFF-GAS
TO DOWNSTREAM PROCESSING

FIGURE 8 Fermentation air applications. *Source:* Courtesy of Pall Corporation.

An early filtration approach, packed towers were employed widely in the industry. Since the early 1980s filtration technology has advanced (Conway, 1984) and there has been an on-going trend to replace depth filters with hydrophobic membrane cartridge filters (e.g., Hoffman-La Roche: Perkowski, 1983; E. R. Squibb and Sons: Bruno and Szabo, 1983).

The recommended filters for sterilization of air feeds to fermentors and bioreactors are the hydrophobic membrane pleated filters. The hydrophobic (water repelling) nature of these membranes can provide for bacteria and bacteriophage removal with 100% efficiency under moist or dry operating conditions. This is an important benefit over fiber glass towers and cartridges. Filters for sterile air feeds should have a 0.2 μm bacterial rating in liquids and a 0.01 μm particulate rating in air service.

For some fermentation the requirement may be for the filtration of fermentor air at an elevated temperature. If an application involves hot air and a longer service life is desired, then a filter that can withstand the elevated temperature is required. Specially designed High Temperature filters are available and can be used in continuous service at a temperature up to 120°C. These filters have a 0.2 μm microbial rating in liquid service and a particulate removal rating of 0.01 μm in gas service. The filter membrane is made of inherently hydrophobic PTFE and the cage, core, and endcaps are specifically designed for high temperature applications.

Sparging

Sparging acts to disperse air evenly in the fermentor or bioreactor containing the growth media and product. The product of choice for this application is porous stainless steel sparging elements, which can provide an exceptionally uniform and fine aeration gas dispersion. These elements are fabricated with one face of porous metal and one face of solid metal. If both surfaces of the sparging elements were porous, bubbles from the under surface may coalesce with bubbles from the top surface.

Porous stainless steel sparging elements should be positioned horizontally in the fermentation tank, with the porous stainless steel facing upward. Fine grades of porous stainless steel (e.g., 3.0 μm absolute liquid rated) are ideal even for sheer sensitive mammalian cell cultures because of their high gas transfer and low shear aeration capability. Elements are typically available in standard and custom designs.

Filtration of Enriched Air or Pure Oxygen for Bioreactors

Modern aeration concepts are increasingly using enriched or pure gaseous oxygen to improve cell culture productivity. Sterilizing grade gas filters with integrity testability by means of a water intrusion test (WIT) are the preferred choice for preventing spoilage of bioreactors by organisms and contaminants in the incoming and outgoing air and oxygen streams.

However, many materials such as organic matter, plastics or even metals can potentially ignite when in contact with oxygen, particularly if also subjected to static discharges, high temperatures, pneumatic shocks or mechanical impact. In addition to the above safety aspects, oxygen or enriched oxygen gases can lead to accelerated oxidation or corrosion of component materials. For that reason, installations for gaseous oxygen including those for filtration need a dedicated risk assessment prior to use. There may also be mandatory requirements in some countries. For example there are special safety requirements in Germany when the oxygen content is over 70% (UVV

Unfallverhütungsvorschriften Sauerstoff, VBG 62, Accident prevention guideline 62 "Oxygen").

Sparged air often contains oil from the compressor which, if mixed with oxygen in a common transfer pipe, presents a potential fire hazard. Therefore, air from compressors must be oil free or use oxygen suitable oils

If any oils or liquid hydrocarbons are present and collect on the filter, they can ignite spontaneously (especially in a hot system) or be ignited by a spark. A filter membrane should therefore be free of flammable residues to avoid fire hazards. Oils are available specifically for use in oxygen service

German UVV/6: "Impurities should be ruled out," therefore avoidance of organic traces as combustible substrate on the filter surface:

- single-use of sterilizing grade gas filters recommended;
- usage of "clean" and organic traces free filters;
- special instructions on filter installation and operation required;
- oil, aerosol and particle free pressurized air line can be supported by usage of coalescers and stainless steel and particle filters.

Static discharge is not too common and at low flow rates, the risk of static is small. However, with high flow rates in dry gases (or low humidity), static risks are higher. It is recommended to ground the housing.

However, static discharge has been observed in dry air systems even at flows of 200 Nm^3/hr (120 cfm) or less per module. The housing size, grounding, tie rod position and number, and other factors play a role.

To avoid static discharging that may lead to membrane damage, ignition and combustion of filters, the follow are recommended:

- grounding of housings;
- only low flow rates (ca. 85 Nm^3/h per 10 inch) by generous sizing; and,
- minimized linear velocities by generous sizing.

German UVV/10 recommends: "The linear velocity, counted as the maximum flow at the lowest operating pressure should be below 25 m/s at operating pressures of 1 to 40 bar g and 8 m/s at operating pressures of more than 40 bar g."

Exhaust Air/Off Gas Filtration

The purpose of a vent filter on a sterile fermentation tank is two fold: to prevent contamination of the tank and to provide containment of the material inside the tank. Prevention of contamination in the tank is desirable for processes that involve long fermentation cycles or require a sensitive fermentation medium (e.g., tissue culture medium). Genetic engineering techniques, as well as fermentation of pathogenic organisms (such as organisms used for the manufacture of vaccines), have made it necessary to protect the environment and prevent the escape of microorganisms from the fermentation tank. The exhaust filtration system for a recombinant or mammalian cell fermentor/bioreactor must yield sterile air to the environment and provide a sterile barrier to prevent ingression of contaminants. Additionally it must be in-situ steam sterilizable and typically has a clean differential pressure less than 1 psid.

The removal efficiencies for simple depth filters (as described above) are typically poor under wet or variable flow conditions. Therefore, membrane filters are recommended for vent filtration applications.

The fermentor or bioreactor exhaust gas line can be contaminated with microorganisms or cells, growth medium components expelled from the fermentor/bioreactor as droplets or as solid particles, and aerosol condensate droplets formed during cooling of the gas in the exhaust system. These aerosol droplets, when present, can potentially block the final filter and must be removed prior to reaching the final filter. Mechanical separation devices, for example, cyclones, condensers, and demisters, may not achieve effective aerosol removal below 5 μm (Porter, 1973). Removal efficiencies and pressure drops also vary significantly with flow rate in such equipment. Recent studies have shown that aerosols in exhaust lines are predominantly in the very fine 1–5 μm range (Jaenchen, 1989).

The contaminants present will depend upon the fermentation conditions, the growth medium, and the design of the exhaust gas system. The basic requirements for a vent filter are: ability to provide sterility, a low pressure drop and in situ steam sterilizable (Fig. 9).

The recommended exhaust filtration system design entails two stages using a polypropylene pleated depth filter cartridge as a prefilter to a 0.2 μm absolute rated hydrophobic membrane pleated filter cartridge. The purpose of the prefilter (typically 1.2-μm-rated) is to remove aerosolized particles and liquid droplets containing cells and/or growth media from the fermentation off-gas or exhaust air. This serves to extend the

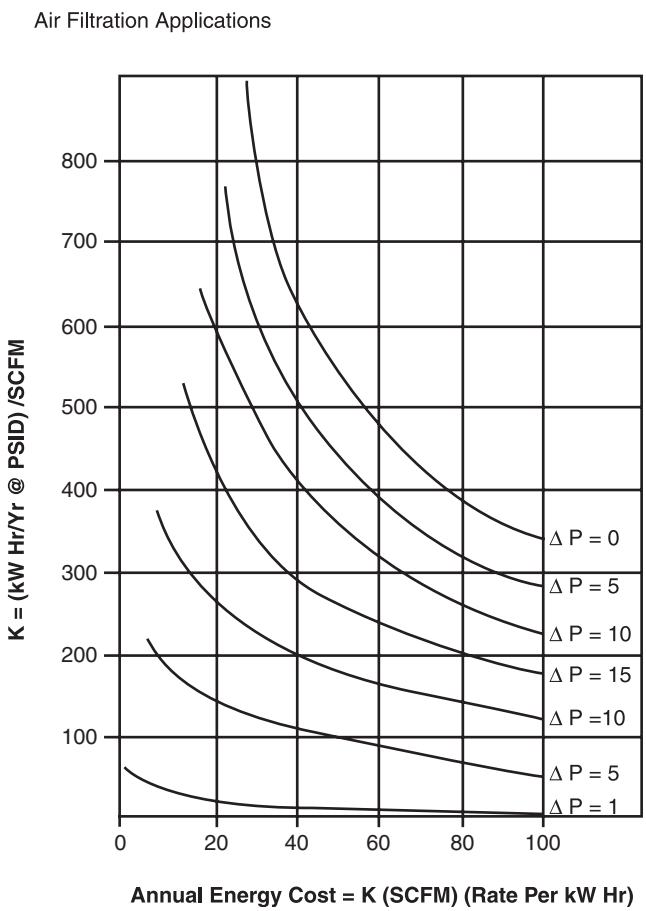


FIGURE 9 Annual energy consumption due to pressure drop K vs. psig.

service life of the final sterilizing filter. If the medium contains only fully dissolved components, such as with a sterile filtered cell culture medium, and if the fermentation is run at low temperatures (< 30°C) and low aeration rate (1–1.5 VVM), the prefilter may be optional.

Like the final sterilizing filter, the pleated polypropylene prefilter should be multiple steam sterilizable. As additional benefit of the prefilter is to act as a coalescer to retard "foam-outs" from reaching the final sterilizing filter.

Sterilizing grade 0.2 μm absolute rated hydrophobic membrane pleated final filters, with PVDF or PTFE membranes, can prevent organisms from entering or leaving the controlled reaction zone, even in the presence of water droplets and saturated gas (Conway, 1984a; Bruno and Szabo, 1983). Steam sterilizability and integrity test values correlated to microbial retention studies under "worst case" liquid challenge conditions provide the highest degree of assurance performance. Redundant systems using a second 0.2-μm-rated sterilizing filter in series are recommended for high risk recombinant organisms.

Condensate control is usually the most critical consideration for this application. In cases in which there is condensate accumulation and if the fermentor is operated with over pressure in the fermentor head, the amount of condensate accumulation can be reduced if a pressure control valve is placed at the fermentor exit, upstream of the exhaust gas filter. An alternative technique for the prevention of condensate accumulation is to use a heating section in the exhaust gas pipe upstream of the filter installation. This can be also be accomplished by specifying steam jacketing on exhaust filter housings. In this case the exhaust gas temperature at the terminal filter must lie above the temperature of the exhaust gas at the fermentor exit. The heater must be properly sized based on the process parameters.

Air Delivery System Improvements

The specific changes required during the retrofit process to convert from fiberglass packed towers to filter cartridges are dependent upon the condition of the existing facility. The upstream requirement to operate cartridge filters is the absence of condensate water and fine particulate material from the feed stream. Since condensate water is a more critical problem for the hydrophilic glass wool media than the hydrophobic cartridges, the existing approach (such as a coalescer or cyclone) may be sufficient. In some installations, a coalescer alone has been shown to be also adequate to remove fine particulate material without requiring a separate prefilter.

In dry air systems utilizing oil-free compressors, a coalescer typically is not required. In such cases, a dedicated air particulate prefilter such as the upstream of the filter cartridges has also been used.

There must also be provision made to eliminate the generation of particulate contaminant in the region between the prefilter and the final filter. Stainless steel piping is recommended for this application. An evaluation of the existing system should be made in order to determine the best approach to minimize the extent of contamination which may reach the final sterilizing grade filters.

Improvements may also be possible in filter design. Most filter cartridges and housings now used were originally designed for liquid filtration applications with gas as a secondary concern. Advances in technology and materials may permit improvements in air filtration system designs that will improve the overall economics of the bioreactor and fermentation processes.

Downstream Processing: Air Filter Applications

Starting with the cells and conditioned broth medium from the fermentor or bioreactor, the objective of downstream processing is to produce a highly purified, biologically active protein product, free of contaminants such as endotoxins, bacteria, particles or other biologically active molecules. This phase of bioprocessing typically comprises a series of unit operations including cell and cell debris separation, fluid clarification and polishing, concentration and purification, and membrane filtration sterilization of the purified product (Table 2).

Cartridge filters are used in many stages of downstream processing, involving filtration of both the harvest fluid and product intermediates, as well as filtration of air and gases required throughout the process. Air filtration applications include vacuum break filters for lyophilizers, sterile nitrogen blankets, tank vents, and sterile air for container cleaning.

Downstream Filtration of Air and Gases in Venting Applications

Absolute rated cartridge filters eliminate contaminants and impurities from air, nitrogen and other gases used in downstream processing, to prevent contamination of product and further protect concentration and purification equipment. Vent filtration ensures containment and freedom from product contamination during fluid transfer operations and protects processing equipment during sterilization cycles.

In fermentation, cartridge filters are used to maintain the sterility of the makeup water, feeds, additives, media in holding tanks, and in fermentor/bioreactor exhaust. Cartridge filters are typically used in downstream processing for the filtration of air, gases and venting applications when it is necessary to vent tanks during fluid transfers, pressurize tanks using inert gases such as nitrogen and argon, protect vacuum lines, sterile vent holding tanks and lyophilizers, for gas purging, blanketing, drying, and when sterilizing equipment by in-situ steaming or autoclaving.

The recommended filters for non-sterile particulate removal applications are polypropylene pleated filters. Hydrophobic membrane pleated filters such as PVDF or PTFE are recommended for aseptic processing. The absolute removal rating for the latter filters should be 0.2 μm determined under liquid flow conditions.

Figures 10, 11 and 12 illustrate steps that can be required in downstream processing applications. The following discussion is on the air or gas filters required in these applications.

TABLE 2 Filter Recommendations for Downstream Processing Air Applications

Application	Filter type	Micron rating	Typical gas flow rate per 10 inch module SCFM
Sterile nitrogen blanket	Hydrophobic membrane	0.2	75–100
Tank venting	Hydrophobic membrane	0.2	75–100
Vacuum break	Hydrophobic membrane	0.2	75–100
Sterile air for container and closure cleaning	Hydrophobic membrane	0.2	75–100

Figure 10 illustrates a cell and cell debris separation and clarification process, which is broken into a primary separation, secondary separation and a cell concentrate section. During primary separation, a cyclone can be used for particle removal; a sterilizing air filter can be used as a vent on the cyclone. A variety of holding and receiving tanks can be employed during the separation and clarification process; these tanks can be fitted with sterile vent filters. During secondary separation, a nitrogen blanket may be needed; the nitrogen gas can be sterile filtered with a hydrophobic filter.

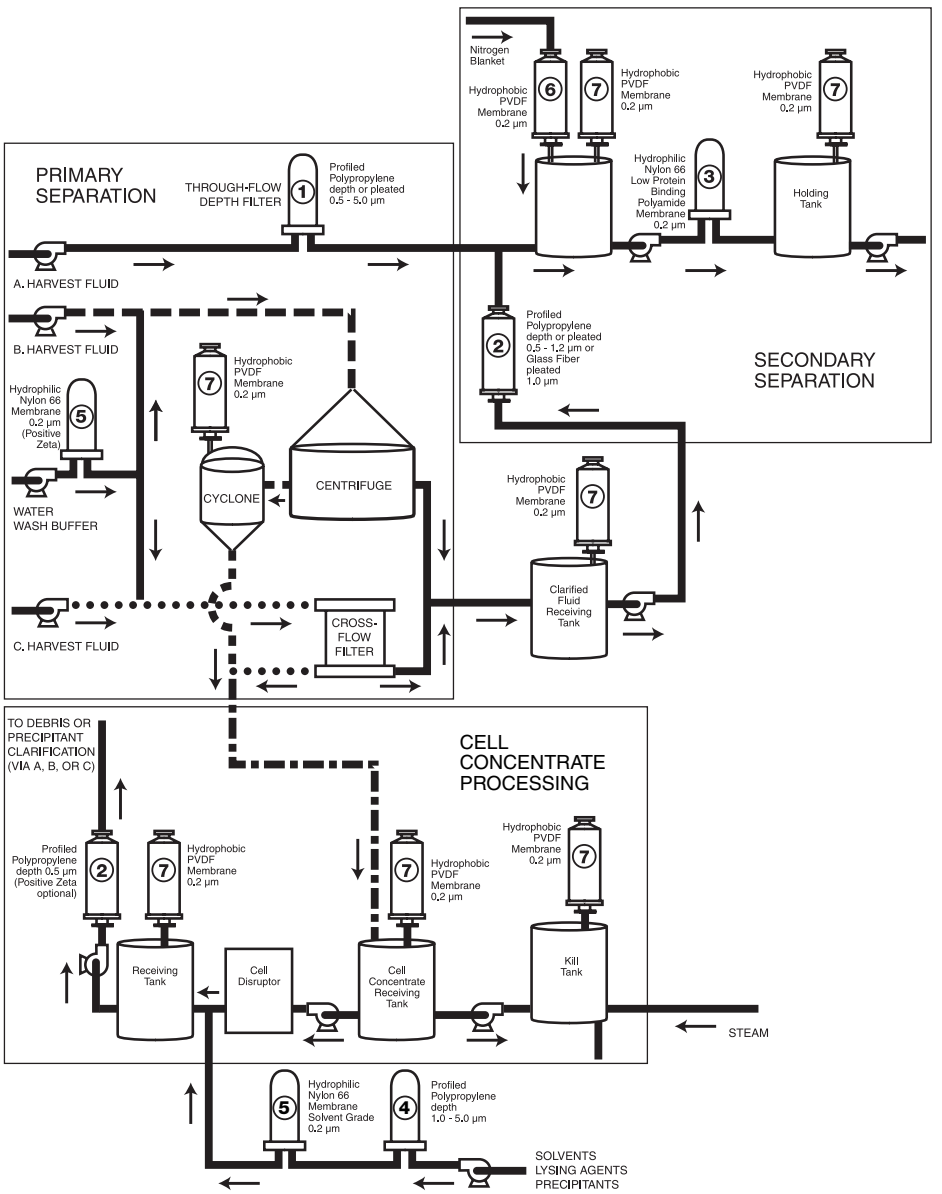

PRIMARY SEPARATION
THROUGH-FLOW DEPTH FILTER
Profiled Polypropylene depth or pleated 0.5 - 5.0 µm
A. HARVEST FLUID
B. HARVEST FLUID
Hydrophilic Nylon 66 Membrane 0.2 µm (Positive Zeta)
WATER WASH BUFFER
Hydrophobic PVDF Membrane 0.2 µm
CYCLONE
CENTRIFUGE
C. HARVEST FLUID
CROSS-FLOW FILTER
Nitrogen Blanket
Hydrophobic PVDF Membrane 0.2 µm
Hydrophilic Nylon 66 Low Protein Binding Polyamide Membrane 0.2 µm
Holding Tank
Profiled Polypropylene depth or pleated 0.5 - 1.2 µm or Glass Fiber pleated 1.0 µm
SECONDARY SEPARATION
Clarified Fluid Receiving Tank
TO DEBRIS OR PRECIPITANT CLARIFICATION (VIA A, B, OR C)
CELL CONCENTRATE PROCESSING
Profiled Polypropylene depth 0.5 µm (Positive Zeta optional)
Receiving Tank
Cell Disruptor
Cell Concentrate Receiving Tank
Kill Tank
STEAM
Hydrophilic Nylon 66 Membrane Solvent Grade 0.2 µm
Profiled Polypropylene depth 1.0 - 5.0 µm
SOLVENTS LYSING AGENTS PRECIPITANTS

FIGURE 10 Air filtration applications in cell and cell debris clarification and purification processes. *Source*: Courtesy of Pall Corporation.

Figure 11 shows a general process for concentration and purification of clarified harvest fluid. Applications for air filtration include:

- vent filters for solvent or buffer tanks;
- vent filters for holding or buffer tanks needed for ultrafiltration and chromatography.

The final pharmaceutical product will often need to be packaged. Figure 12 shows a generalized filling operation.

A sterile nitrogen blanket and thus a sterilizing grade hydrophobic filter may be needed.

- Sterile air or nitrogen may be needed for container cleaning.
- A vent filter can be used on holding tanks.

Several specific applications are discussed in the following sections.

Vacuum Break Filters

Sterilizing grade filters are employed in freeze dryer installations to filter the gasses used to maintain the chamber pressure and to break vacuum during operation and in sterilizers for vacuum break purposes.

As an example, the operation steps required for a sterilizing filter used in a lyophilizer as a vacuum break is described below. The system is illustrated in Figure 13.

1. *In situ steam sterilization*: The filter assembly and the receiving vessel and vent filter attached on the vessel are in situ steam sterilized
2. *Cooling*: After the sterilization, the steam is shut off and air or nitrogen is immediately added to the system. Air or nitrogen gas are used to prevent a collapse situation (reverse pressurization - see below) and to cool the system and filter.
3. *Addition of integrity test fluid*: The wetting fluid for a Forward Flow integrity test, which is typically an alcohol water solution is placed in the wetting solution feed vessel. Compressed air or nitrogen pressure can be used to flow liquid through the filter cartridge in order to wet the membrane for an integrity test. The wetting fluid is collected in a receiving vessel.
   Alternatively, water could be introduced on the upstream side for the performance of a WIT.
4. *Integrity test*: An filter integrity test instrument is connected for the performance of the integrity test. The use of an automated instrument allows for remote operation. If the filter fails the integrity test, then the filter should be changed.
5. *Drying*: After the test, the membrane filter is dried to remove the wetting fluid (or water if an intrusion test has been employed). Compressed air or nitrogen can be used.
6. *Lyophilization*: At this point the system is ready for the lyophilization operation.

Blow–Fill–Seal

Blow–fill–seal equipment can be used for the aseptic filling of pharmaceutical products. The container is formed and seal aseptically. Air filtration is required to assure sterility in this unit operation. A typical arrangement is illustrated in Figure 14.

For buffer tank air hydrophobic membrane filters are used to supply sterile air to a buffer tank on the blow fill seal machine. This blanket air is used to drive the sterile solution through a pneumatically controlled dosing system. The air used in the buffer tank

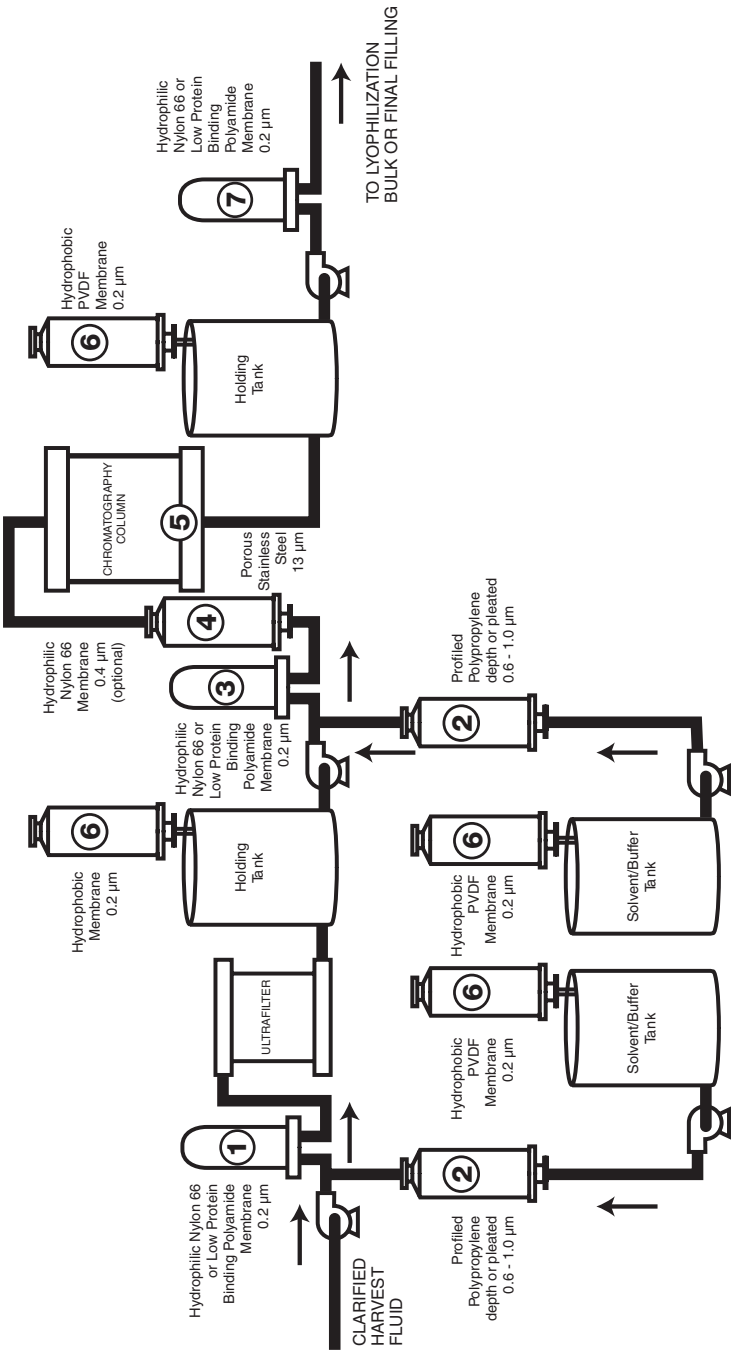

CLARIFIED HARVEST FLUID
Hydrophilic Nylon 66 or Low Protein Binding Polyamide Membrane 0.2 µm
1
Profiled Polypropylene depth or pleated 0.6 - 1.0 µm
2
ULTRAFILTER
Hydrophobic Membrane 0.2 µm
6
Holding Tank
Hydrophilic Nylon 66 or Low Protein Binding Polyamide Membrane 0.2 µm
3
Hydrophilic Nylon 66 Membrane 0.4 µm (optional)
4
CHROMATOGRAPHY COLUMN
5
Porous Stainless Steel 13 µm
Hydrophobic PVDF Membrane 0.2 µm
Hydrophilic Nylon 66 or Low Protein Binding Polyamide Membrane 0.2 µm
7
TO LYOPHILIZATION BULK OR FINAL FILLING
Solvent/Buffer Tank

FIGURE 11 Air applications in the concentration and purification of clarified harvest fluid. *Source*: Courtesy of Pall Corporation.

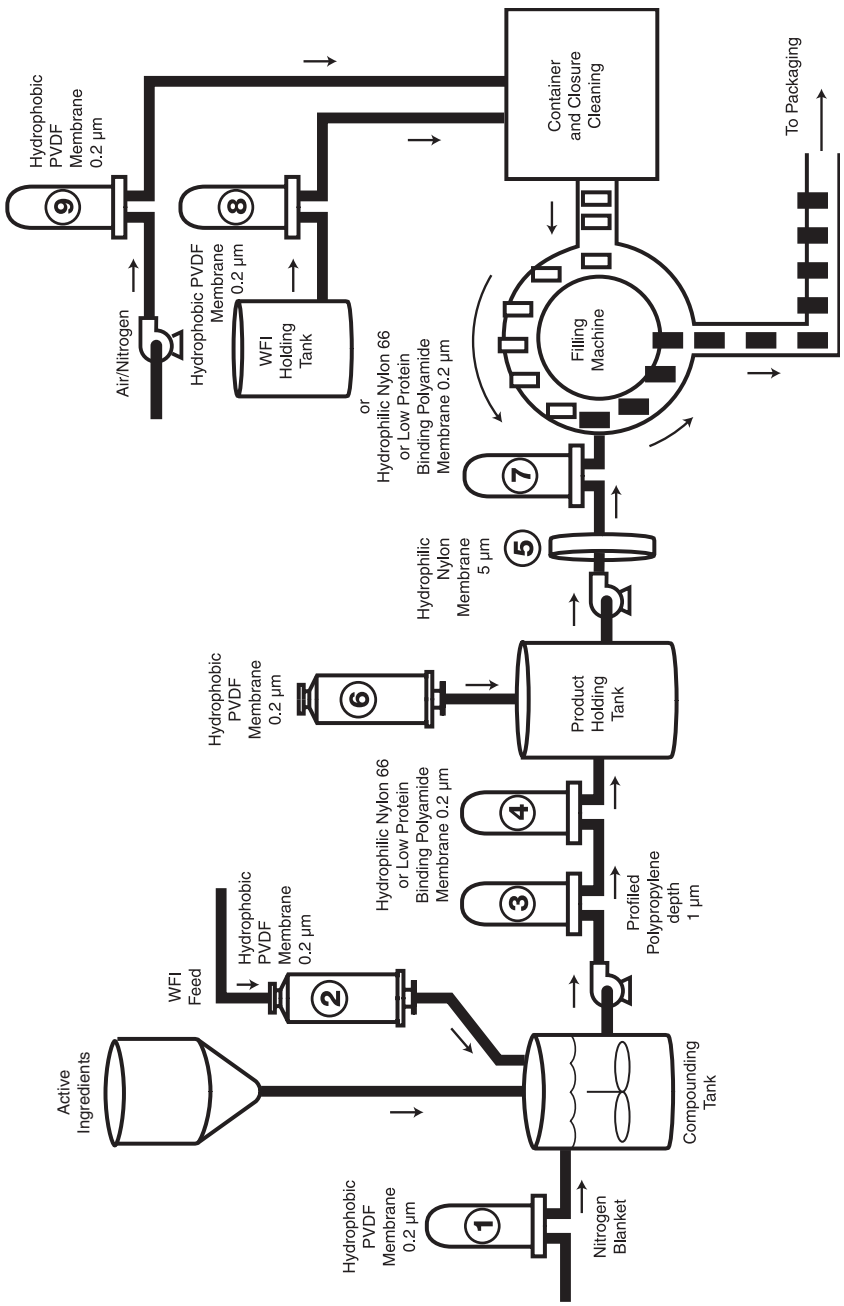


FIGURE 12 Air filtration applications in a filling process. *Source*: Courtesy of Pall Corporation.

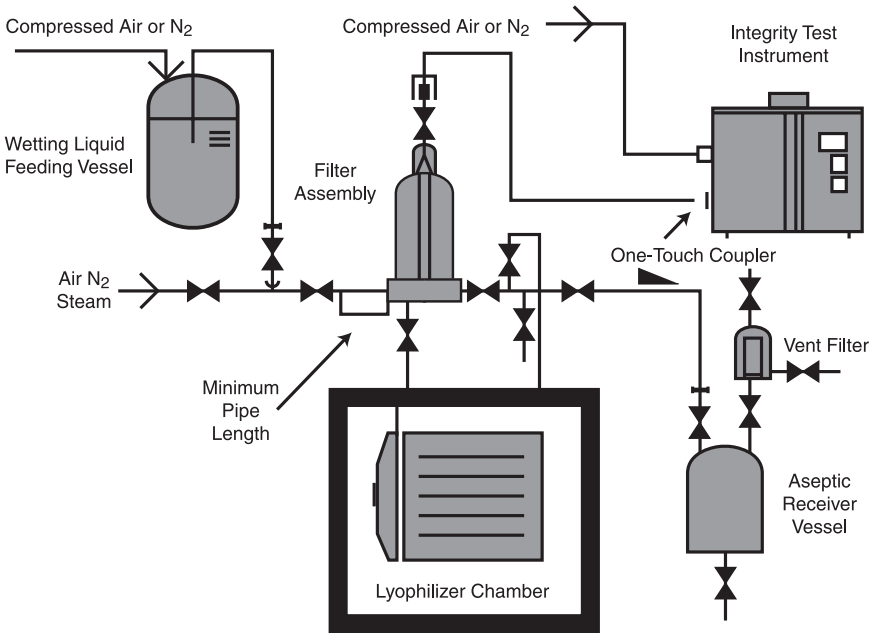


FIGURE 13 Air filtration applications in a Vacuum Break application (lyophilizer).

is referred to as the gas cushion or buffer tank air. Hydrophobic membrane filters provide sterile air used to form the hot moldable plastic tube (parison).

The air used to form the parison is known as the parison support air. The Parison is subsequently blow molded into the shape of a ampoule strip or a bottle.

Typical requirements for the filters used include that the filters be steam sterilizable, integrity testable, and the proper size to prevent restriction of gas flow. Hydrophobic membranes are used to prevent wetting out and to maintain high flow rates even in moist conditions.

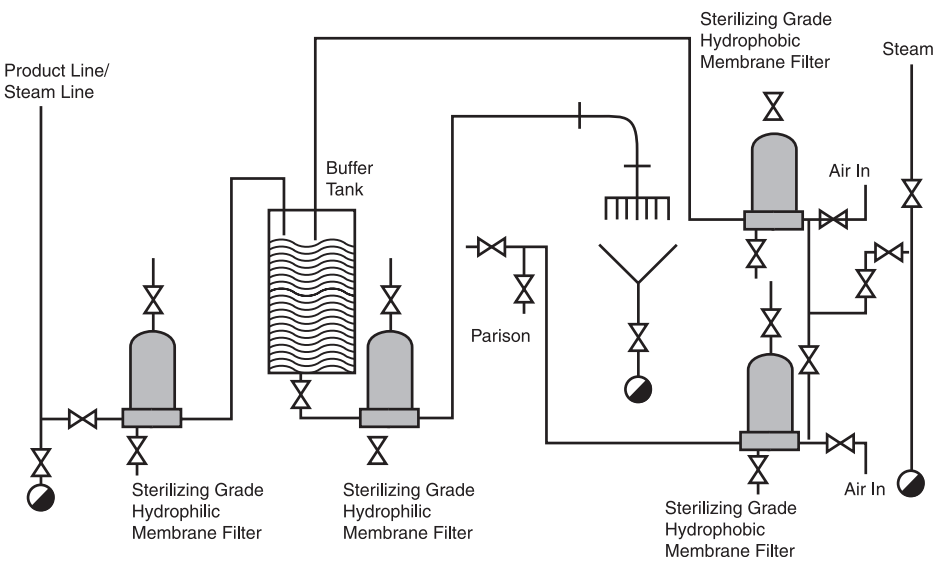


FIGURE 14 Air filtration applications in a blow–fill–seal operation.

Utilities

There are a number of peripheral unit operations required during a sterile process. The air filtration applications will be described for these applications.

Hot and Ozonated Water Tanks and Systems

Many pharmaceutical processes require large volumes of water. It is critical that the pharmaceutical grade water used is protected from particulate or microorganism contamination to ensure that the process operations do not become inadvertently contaminated. There are several approaches that can be used to ensure that the water remains free from contamination, including the storage of Purified Water, Highly Purified Water or Water for Injection, at a minimum temperature of 80˚C (176˚F) to discourage microbial growth in the storage system. Another approach is to add ozone, which acts as an antimicrobial and oxidizing agent, to ambient as well as to hot water storage and distribution systems. Usually, the water is stored in tanks fitted with a sterilizing grade vent filter to ensure that the tank can be properly vented for filling and emptying without the risk of a secondary contamination from the tank environment.

It is known that standard vent filters can show evidence of oxidative degradation of the polypropylene components from exposure to ozone after three to six months in hot and ozonated water tank applications. The polypropylene drainage layers became brittle and powdery due to oxidative attacks from the ozone or the hot air above the water. This type of degradation can lead to:

- material and particle input into WFI or pure water storage tanks;
- passage of microorganisms through damaged cartridge components;
- failure of cartridge integrity.

To provide a longer vent filter service life in applications that involve a highly oxidative environment PTFE membrane filters have been developed with a special support and drainage material which is more robust in oxidative environment. In addition, the polypropylene hardware used for these filters has been optimized to have greater resistance oxidation after longer exposure times. For this reason, the use of standard vent filter cartridges in elevated temperature oxidative conditions requires a frequent change out of filter cartridges in order to avoid degradation of the filter materials.

Sterilizing grade membrane filters can be used in vent applications in which the fluid in the tank is at an elevated temperature. One such application is the vent used to prevent contamination in a water for injection tank. The water is at 80 ˚C or higher. When a sterilizing grade air filter is used for this type of vent service, a steam jacketed housing is typically used. It is only necessary to maintain the temperature of the filter cartridge at a temperature slightly above the dew point of the vapor. The steam introduced into the jacket should be at ambient pressure. Continuous operation of the jacket at a significantly higher steam pressure and temperature can reduce the service life of the filter due to accelerated aging of the hardware by oxidation.

Aging of the filters by oxidation depends on the status of the system. Oxidation does not occur when the cartridge is being steamed, since there should be no air present in a properly operating steam-in-place system. If a cartridge is exposed to air at an elevated temperature, oxidation of the material in the filter, such as polypropylene hardware, will be accelerated. Oxidation will also occur when the filter is in a stagnant situation, that is, it has no air flow going through it. The flow of air through a filter can moderate the temperature environment, whereas under stagnant conditions the temperature of the filter

will rise to the temperature of the housing. Stagnant conditions can exist when the tank is not being used or when the tank is empty. To prolong service life, we recommend that the steam jacket is turned off when there is no air flow through the filter for extended periods of time, when operating conditions permit.

For any application for vent filtration when the temperature of the air is above 60˚C and/or ozone is present the filter used either has to be specifically designed for this application or should be inspected very frequently to track filter life and to detect the start of the oxidative damage process to set change-out parameters.

Hot Air

If an application involves hot air and a longer service life is desired, then High Temperature filters can be used. High Temperature filters can be used in continuous service at a temperature up to 120˚C. These filters have a 0.2-μm microbial rating in liquid service and a particulate removal rating of 0.01 μm in gas service. The filter membrane is made of inherently hydrophobic PTFE and the cage, core, and end caps are specifically designed for high temperature applications. For hot dry air, high temperature filter cartridges are expected to have a service life of up to 1 year at 100˚C. However, change-out should be set for actual service conditions.

Disposable Filtration Systems

Integrated single use bioprocess systems incorporating filters, tubing, bags, mixing systems and aseptic connection devices can play an integral role in helping companies bring their drug products to market faster than ever before. Single use systems can be scaled up faster than their stainless steel counterparts and offer process flexibility every step of the way, in addition these systems offer a wide range of benefits such as:

- eliminating cleaning and cleaning validation;
- eliminate sterilize in place (SIP) system sterilization and validation;
- eliminates cost of CIP including use of caustic chemicals, detergents and WFI consumption;
- efficient use of space: smaller over-all footprints and reduced storage requirements;
- reduction in cross contamination;
- reduce down-time associated with stainless steel assembly/labor;
- ability/flexibility to increase production capacity.

Most of these systems are provided pre-sterilized by gamma irradiation. For the filtration of inlet air and vents associated with these systems, the filter capsules should contain gamma stable membrane and hardware. There are three commonly used hydrophobic membranes, PES, PVDF and PTFE, manufactured as capsules in polypropylene hardware. However while PES and PVDF are resistant to gamma irradiation sterilization, PTFE is much less resistant and levels above 25 KGy can damage these filters. In addition, the hardware should be capable of being sterilized to the required level and the filters should be fully validated for gamma use.

Depending on, where these systems are used (R&D, production, etc) the ability to integrity test these capsules without the use of solvents can also be a benefit.

The vent filters used in disposable systems will require an integrity test but these systems have a limitation on the use of solvents such as alcohol because there is no easy way to eliminate the solvent from these disposable systems. The best integrity test for these vents is the WIT because it uses WFI which does not enter the system.

Steam Filtration

Process equipment and final filters are frequently sterilized by direct steam flow in situ, during the normal line sterilizing cycle. This eliminates the need for making aseptic connections and risking recontamination. Filtered steam is required for this SIP of filters, piping, vessels and filling equipment. Steam is also required for general equipment cleaning and sterilizing. The steam often contains significant amounts of pipe scale and other corrosion products. This particulate material should be removed in the interest of overall cleanliness and to avoid burdening the prefilters and final filters.

Particulate contamination in process steam is efficiently retained by porous stainless steel filters with an absolute gas rating of 1.2 μm. Porous stainless steel filter assemblies are typically sized at steam flow rates of 30–40 ACFM per square foot of filter medium.

General Considerations for Operation

Integrity Test Considerations and Guidelines

Recommendations on When A Filter Should be Integrity Tested. The U.S. Food and Drug Administration (FDA) June, 1987 "Guideline on Sterile Drug Products Produced by Aseptic Processing" (pp. 30–31) states the following:

> After a filtration process is properly validated for a given product, process, and filter, it is important to assure that identical filter replacements (membrane or cartridge) used in production runs will perform in the same manner. One way of achieving this is to correlate filter performance data with filter integrity testing data. Normally, integrity testing of the filter is performed after the filter unit is assembled and sterilized prior to use. More importantly, however, such testing should be conducted after the filter is used in order to detect any filter leaks or perforations that may have occurred during the filtration.

Field experience with the integrity testing of sterilizing grade filters shows that various combinations of integrity test procedures are in use and that different testing schedules, both pre- and post- use, are followed. Perhaps the best approach is to perform the test or combination of tests that provide the highest degree of accuracy commensurate with the economics and the practicalities of the process.

The following are possible approaches to integrity testing the filters:

1. do not test filters and base filter change out on historical data,
2. test filters only upon installation,
3. test filters after the first sterilization,
4. test filters after every use,
5. test filters in situ,
6. test filters in a laboratory,
7. have parallel filters so that while one filter is in use the other filter can be tested and prepared for use,
8. use redundant filters.

Since the requirements for the use and testing of sterile gas filters can depend on location and application, several different types of filter tests can be used. Filter users typically employ the Forward (Diffusive) Flow or Pressure Decay tests, which require the use of a low surface tension solvent, such as 60/40 IPA/water or 25/75 t-butyl alcohol/water. This is a well proven, widely accepted approach, which is directly correlatable to bacterial challenge.

Air filters are typically integrity tested when there are installed and retested periodically based on service conditions and operating requirements (e.g., once a month). Change-out schedules based on filter life studies have also been used in conjunction with integrity testing. Because filters in these applications are often sterilized in situ and can be damaged during in situ steam sterilization by reverse pressurization if the sterilization procedure is not properly controlled, filter life study data or a periodic integrity test regimen can be used only if the filter sterilization procedure is validated and in control.

In certain cases (such as in facilities in which the use of a non-flammable fluid is required, or when disposal of organic solvents is a concern) some filter users in aseptic processes have recently considered the use of water-based tests (e.g., WIT) for hydrophobic filters.

In summary, there are a number of integrity tests possible for a hydrophobic air filter. The selection of the appropriate test and the appropriate test schedule depends upon the specific application.

Drying of Filter Cartridges used for Air Filtration. After an integrity test has been completed, it is typically desirable to remove the wetting fluid from the filter. This can be accomplished by blowing clean, dry (−40˚C dew point) air or nitrogen through the filter. It is necessary to qualify this procedure, since every system is different.

One way of qualifying this procedure would be to first weigh a dry filter in housing. As a starting point, 75 cubic feet of dry air per square foot of filter area should be blown through the filter. A minimum initial pressure of at least 25 psi (so that bubble point is exceeded) will be required. The vent on the housing can be opened slightly to facilitate rapid drying of the housing and filter membrane. As flow is initiated the pressure can be reduced if desired. The filter and housing can then be weighed to determine if the weight is the same as the weight of the dry filter and housing. Additional air can be blown through the filter until the weight matches the weight of the dry filter and housing. A safety factor can be added on the drying cycle if required.

Air filters which have been water wetted can also be dried by placing filter in an oven at an elevated temperature for an appropriate period of time. Filters that are alcohol wet should be flushed with water prior to oven drying.

The actual cycle time depends upon the filter cartridge style (i.e., cartridge length, closures) as well as the operating conditions of the oven (air velocity, relative humidity). Therefore, the actual drying time should be determined by a test at each installation.

It is important to emphasize that the temperature maximum for the filter must not be exceeded and that the oven environment should not cause degradation to the filter components.

Filter Service Life. Filter cartridge change-out is usually based on actual experience, with a safety factor. Filters should be inspected on a monthly basis for oxidation. This should be supplemented by monitoring the pressure drop across the filters during operation to determine if the filters are plugging, and routine integrity testing to confirm filter integrity during the service life of the filter. Alternatively, filter life studies, with an appropriate safety factor, could also be used to set a change-out schedule. Actual conditions for each application should be used during filter life studies.

Steam Sterilization Guidelines for Sterilizing Grade Membrane Filters

Membrane filters can be sterilized by chemical sterilants (such as ethylene oxide, hydrogen peroxide in vapor form, propylene oxide, formaldehyde, and glutaraldehyde), radiant energy sterilization (such as gamma irradiation) or steam sterilization. The most common method of sterilization is steam sterilization and it will be the focus of further

discussion regarding the usage of steam sterilization for membrane filters used for air filtration.

Basic Procedures. Steam sterilization of a membrane filter can be accomplished either by an autoclave or by in situ steam sterilization. In situ steam sterilization can be effectively accomplished by a variety of different process arrangements. Below is an example of an effective procedure that can be used as a guideline for in situ steam sterilization (Figure 15). Steam sterilization is often the most critical portion of the process and it is important that the procedures followed lead to sterilization of the system and do not impart any damage to the membrane filters.

1. Start with ALL valves closed.
2. Fully open valves V2, V5, V4, and V9.
3. Fully open condensate drains V12 and V13, housing drain V8 and housing vent valve V6.
4. Preset steam pressure to required steam pressure for the Vent Filter Assembly. Then slowly open steam valve V10. After Condensate has been expelled from V12 and V8, close both valves to crack.
5. Partially close vent valve V6 when steam flow is evident. Drain condensate from V13. After condensate has been expelled, close V13 to crack.
6. Permit steam to flow through the system for the required sterilization time, ensuring that no more than 5 psi pressure differential is developed across the filter.
7. When sterilization time is complete, close V10 and drain valves V13, V8, and V12, and vent valve V6.
8. Preset pressure of regulated air or nitrogen at 3 psi above the Sterilization steam pressure. Close steam valve V10 and immediately open air or nitrogen valve V11.
9. Steam may be flushed from the assembly to assist cooling by carefully opening vent valve V6. Close valve V6 after venting.

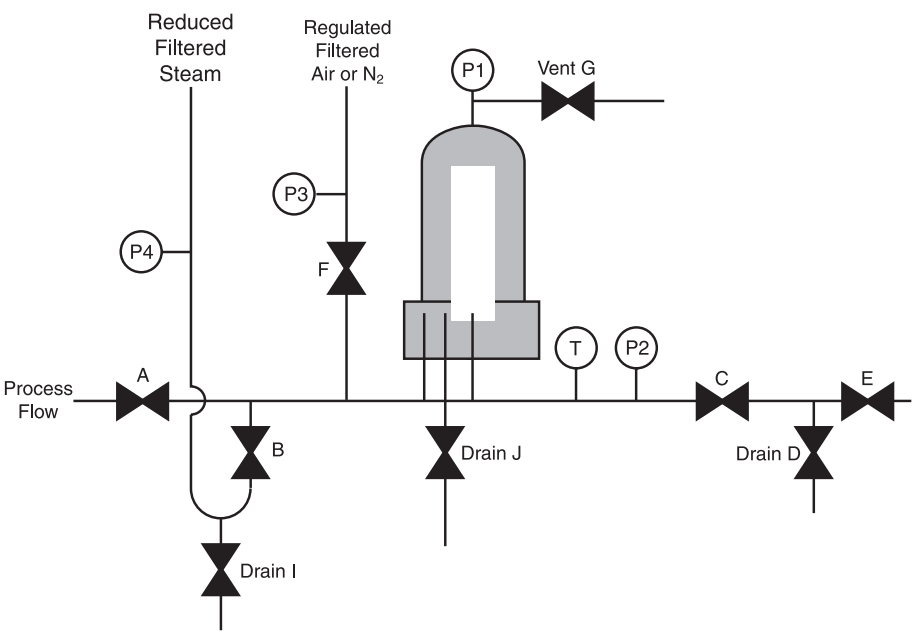


FIGURE 15 Steam sterilization arrangement (Haughnrey, 1995).

10. Allow assembly to cool to ambient or to process temperature.
11. Close air or nitrogen valve V11.
12. Relieve the gas pressure in the filter assembly via vent valve V6. Filter assembly is now ready for use.

Sterilization of Filters and Downstream Equipment

In-situ steam sterilization of filters and downstream equipment can be problematic. Sterilization of equipment assemblies requires very careful control and validation since the required steaming cycle is influenced by the equipment configuration, materials of construction, heat capacity and volume of the downstream system. In addition, the vent filter must be steamed in the reverse direction.

It is always preferable to sterilize the filter and downstream components separately. However, if the filters and downstream equipment are steamed simultaneously, appropriate vacuum relief safety devices must be fitted on downstream vessels which cannot withstand negative pressure without collapse.

Elimination of Reverse Pressurization Conditions During In-Situ Steam Sterilization

At the conclusion of the sterilization cycle, it is recommended that an integrity test be conducted in order to ensure that the filter has not been damaged during the steam procedure. Damage to filter cartridges at this stage has occasionally been experienced. Analyses of such incidents have shown that over 70% of such failures can be attributed to a reverse pressurization condition which developed during the cooling phase of the steam sterilization cycle. The possibility of damage to the filter from exposure to a reverse pressurization condition is increased during steam sterilization since the element is also subjected to elevated temperature conditions.

Reverse pressurization condition can be avoided by following recommended procedures. During in-situ steam sterilization, the steam is introduced into the filter housing in a procedure which displaces the air from the housing and then permits the steam to flow through the filter and exit through the downstream outlet of the system. At the conclusion of the in-situ steam sterilization cycle, the steam valve is closed and air or nitrogen is applied to the system at approximately the same pressure as the steam. This procedure is the critical step in order to prevent reverse pressurization damage.

The most common operator error at this point is to close the steam valve and not to apply the specified air or nitrogen pressure. If the steam valve is shut and a non-condensable gas is not introduced, the housing is essentially an isolated system. Due to differential heat transfer during the cooling cycle, steam condenses to liquid water at different rates upstream and downstream of the filter. As a consequence, pressure differences of 5–15 psi across the wetted filter medium may develop. This condition may exist for only several minutes, however this period is sufficient for permanent damage to be done to the filter.

This situation can be completely avoided by maintaining the pressure through the introduction of a non-condensable gas (air or nitrogen) at the conclusion of the steaming cycle. This step eliminates the conditions for reverse pressurization.

Condensate Control

Condensate can form on filter membranes during the SIP procedure. The following process procedures can be used to minimize the accumulation of condensate on a filter for both air and vent filters.

Plumbing

1. Figure 15 illustrates a recommended system configuration for steam sterilization. Recommended locations of drains are indicated (Drains D, I, and J).
2. Pressure gauges and temperature sensors in the system should be checked on a routine basis for accuracy.
3. Steam traps should be checked periodically to make sure that they are working properly.
4. A temperature sensor should be installed on the downstream side of the filter, to verify that the steam is at the correct temperature.
5. Condensate traps should be located as close to the inlet of the filter housing as possible.
6. The filter cartridge should be positioned so that the open end is facing down in order to permit condensate to drain. The housing should be installed vertically to prevent condensate from accumulating on the upstream side of the filter.
7. Pressure gauges (accurate over the range of 0–45 psi) should be installed both up and downstream of the filter housing, so that the pressure differential (ΔP) across the filter can be monitored. The pressure differential across the filter should not exceed 5 psid. A higher ΔP can cause damage to the filter when it is at elevated temperatures during the steam sterilization process. If a filter has been wetted with condensate and steam is introduced, the ΔP can rapidly exceed 5 psid.
8. It is recommended that the filter housing has a vent valve and a drain valve on the upstream side of the housing for the removal of condensate (Figure 15: Vent G and Drain J). These valves should be partially open during steaming to prevent the accumulation of condensate.

Steam

1. Dry, saturated steam should be used in order to prevent excess condensate from entering the system. Super-heated steam should **not** be used, because it could subject the filter to a high temperature and cause damage to the filter.
2. The inlet pressure of the steam must be checked to verify that the required steam pressure is being delivered to the system, to ensure that the system is properly being sterilized.
3. Condensate should be drained from the steam line before introducing steam flow into the filter housing. This can be accomplished by ensuring that condensate drains in the upstream lines and in the housing are opened and functional so that condensate is removed before steam flow is initiated through the filter.

Steam Cycle

1. During the steaming cycle, the drains and steam traps must be partially opened to prevent a build-up of condensate.

Post Steaming Procedure. A non-condensable gas, such as regulated air or nitrogen, must be introduced into the housing immediately after the steam valve is closed to prevent a vacuum from being formed on the upstream side of the filter. The gas pressure should be 3 psi higher that the steam pressure.

1. The upstream vent valve should be carefully opened when air is introduced after steaming. This will facilitate cooling, by flushing the steam from the system. After venting, the valve may be closed.
2. Condensate can be removed from the membrane by continuing to apply a low air or nitrogen pressure after the steam has been removed from the system. The actual conditions should be validated for the application. This can be accomplished by weighing a dry filter, prior to use. After the filter has been steamed and blown with air, it can be weighted to a constant weight to establish that it has been dried thoroughly. The constant weight should be within a gram of the dry filter. By using this procedure an amount of air required for drying a filter in a particular application can be established. As a starting point, 75 cubic feet of air per 10 inch filter is suggested.

Considerations for Exhaust Gas Filters

Condensate control in vent filter applications is dependent upon the attention of the individual process operator. Many processes are operated according to experience and remove sufficient condensate to avoid filter blockage by condensate. This is evident from field reports. The following suggestions have been found to be useful for reduction of condensate accumulation on exhaust gas filters:

1. Condensation can be minimized in the vent filter housing by initiating steam flow through the vent housing only after the upstream vessel has developed some steam pressure. This will reduce the amount of wet steam entering the vent housing.
2. Water vapor can condense on a vent filter membrane during use if the fluid in the tank is water based and the temperature of the filter is lower than the dew point of the fluid in the tank. A recommended procedure for avoiding of the formation of condensate on the membrane is to maintain the filter assembly at a temperature slightly above the dew point of the liquid in the vessel. Formation of condensate can be prevented by fitting a heating section on the exhaust gas line, upstream of the vent filter. The purpose of the heat exchanger is to ensure that the temperature of the exhaust gas at the filter is above the temperature of the exhaust gas at the fermentor exit. The effect of the heating unit is to lower the relative air humidity from 100% at the fermentor outlet to 20–50% downstream of the heating unit at the filter housing inlet. NOTE: The temperature of the filter should not exceed 60˚C, since this is the maximum temperature for continuous service.
3. This applies to filters that operate with back pressure in the head. Condensate can be reduced on the exhaust filter, if a pressure control valve is positioned at the fermentor exit, upstream of the exhaust gas filter. The condensate can be reduced, since the relative air humidity is lowered.
4. The amount of condensate that can accumulate in a filter is related to the exhaust gas flow velocity. At a higher flow velocity, the amount of condensate will increase. Thus, if there is any way in which the flow velocity of the exhaust gas can be reduced, the amount of condensate can be lowered. One possible solution would be to fit the exhaust gas line with a section of wider diameter piping.

CONCLUSIONS

This chapter has provided information on a variety of air filtration applications: fermentor inlet air, fermentor vent gas, vents on Water for Injection tanks, and vacuum break filters

during lyophilization. In addition to a description of the air filtration applications, guidelines were provided on the usage of the filters in these applications.

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Sterility Testing by Filtration in the Pharmaceutical Industry

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INTRODUCTION

Definition of sterility: The absence of viable and actively multiplying micro-organisms

Test for sterility: The test described in the International Pharmacopoeias which consists in searching for microbiological contamination in a representative sample of a sterile product

In order to comply with the regulations, the test for sterility must be qualified in performance especially when there is a risk of inhibition of the growth of micro-organisms (e.g., test of antibiotics).

Although the test for sterility, as it is described in the current regulations, has statistical limitations, it remains the only microbiological batch release test of sterile products in the pharmaceutical industry.

REGULATORY CONTEXT: DESCRIPTION, ANALYSIS AND INTERPRETATION

International Pharmacopoeias

Knowing the International Pharmacopoeias is essential to understand the Test for Sterility of pharmaceutical sterile products.

There are three major Pharmacopoeias describing the requirements of sterility testing:

1. The United States Pharmacopoeia (USP 29) <71>.
2. The European Pharmacopoeia (EP 5.0) 2.6.1.
3. The Japanese Pharmacopoeia (JP 14). Not discussed here.

These texts have been partially harmonized recently which helps international companies to rationalize the test procedures and validation protocols when exporting to foreign countries where different regulations are in place. Nevertheless, some minor discrepancies still remain.

Environment of the Test

The test environment is not clearly specified in details in the Pharmacopoeias as it is in the FDA Guidance for Industry or the PIC/S Guide to GMP texts (see the following

sections). Nevertheless, it is obvious that the quality of the test environment can impact the result of the test and must be monitored in a proper way to minimize risks of false positive and false negative results.

Sterility test is generally performed in a class A laminar Air Flow cabinet within a class B, in a class A clean room if available or in an Isolator. More and more users take advantage of isolator technologies as it creates a higher assurance that false positive tests don't happen. These enclosed systems have a very high reliance and create a clean environment without user intervention. Any space the sterility test is performed in requires routine air sampling and monitoring for organisms to assure that such airborne contaminants do not influence the outcome of the sterility test adversely.

Culture Media

In order to allow the growth of most of the micro-organisms, yeasts, moulds, aerobes and anaerobes, spore forming and non spore forming organisms, two culture media and several micro-organisms have been selected in the International Pharmacopoeias:

1. *Fluid Thioglycollate medium* allows the growth of anaerobic and aerobic bacteria.
2. *Soybean-Casein Digest medium* is used for the detection of fungi and aerobic bacteria.

In other terms, both media compliment each other and provide an overlap of nourishment for aerobic bacteria in order to optimize the overall recovery of the test.

USP 29 and EP 5.0 clearly define the media compositions as well as a test, called Growth Promotion Test, which consists in growing several strains of micro-organisms in a maximum time period.

The following table, listing the test microorganisms, are compiled from the USP and the EP:

European pharmacopoeia		United States pharmacopoeia	
Aerobic bacteria			
Staphylococcus aureus	ATCC 6538, CIP 4.83, NCTC 10788, NCIMB9518	*Staphylococcus aureus*[a]	ATCC 6538, CIP 4.83, NCTC 10788, NCIMB9518
Bacillus subtilis	ATCC 6633, CIP 52.62, NCIMB 8054	*Bacillus subtilis*	ATCC 6633, CIP 52.62, NCIMB 8054
Pseudomonas aeruginosa	ATCC 9027, NCIMB 8626, CIP 82.118	*Pseudomonas aeruginosa*[b]	ATCC 9027, NCIMB 8626, CIP 82.118
Anaerobic bacteria			
Clostridium sporogenes	ATCC 19404, CIP 79.3, NCTC 532, ATCC 11437	*Clostridium sporogenes*[c]	ATCC 19404, CIP 79.3, NCTC 532, ATCC 11437
Fungi			
Candida albicans	ATCC 10231, IP 48.72, NCPF 3179	*Candida albicans*	ATCC 10231, IP 48.72, NCPF 3179
Aspergillus niger	ATCC 16404, IP 1431.83, IMI 149007	*Aspergillus niger*	ATCC 16404, IP 1431.83, IMI 149007

[a] or *Bacillus subtilis* as an alternative.
[b] or *Micrococcus luteus* (Kocuria rhizophila) as an alternative.
[c] or *Bacteroides vulgatus* as an alternative when a non spore forming microorganism is desired.

The slight discrepancy between the EP and the USP probably lies in a separate official FDA Code of Federal Regulation 21 CFR 610.12 where different microorganisms are listed.

In the 21 CFR 610.12 *Pseudomonas aeruginosa, Staphylococcus aureus*, and *Aspergillus niger* are not listed and only one ATCC number is defined for each microorganism.

This might be of importance for Pharmaceutical companies selling their drugs in the USA, whether they produce in the USA or anywhere else in the world.

A special note states that the "... viable microorganisms used for inoculation are not more than five passages removed from the original master seed lot." As a matter of fact, old cultures might not grow as well as younger ones.

Sampling

Sampling parameters like the number of items to be tested in each batch of product and the quantity of product in each container are clearly defined in the Pharmacopoeias.

The rules are based on the size of the batch, the volume of the container and the type of product. In general, the bigger the batch size, the higher the number of containers to be tested. Nevertheless, over a batch size of 500 items, which is very often the case, the number of items to be tested doesn't exceed 10 or 20 depending on the type of product. Users generally do not take more samples although this is perfectly allowed. Quantity of product to be tested per container depends on the volume of product in each container and the type of product. In general, when the total volume of product in the container is small, the whole content is tested. When the total volume is large, only a part of it is tested. Specific rules apply depending on the nature of the product to be tested.

Validation Test

The validation test is based on the incorporation of selected microorganisms (the same strains used for the growth promotion test) during the sterility test procedure in order to check whether they will grow in a visually comparable way to a positive control. In other terms, this test demonstrates that the procedure of sterility testing does not affect the growth of microorganisms. For instance, if the product to be tested has inherent antimicrobial activity (e.g., antibiotics or drugs containing preservatives), it is important to demonstrate that the established procedure does not allow a risk of inhibition of the growth of the referenced microorganisms. The antimicrobial activity is removed using an appropriate rinsing procedure or neutralized (e.g., using of β-lactamase for neutralising the activity of penicillins or cephalosporins).

The accuracy of this validation test is certainly not optimal as it mainly consists in comparing turbidity in the test and positive control containers. As a matter of fact, it is not clearly demonstrated that a moderate microorganism inhibition will be identified by comparing turbidity in a liquid culture media. On the other hand, the microorganisms listed in the USP29 and EP5.0 are not selected based on their specific sensitivity to a defined antimicrobial substance, sterilising agent or antibiotic. Then, it might be informative to select sensitive microbes for running validations studies depending on the type of antimicrobial agent in the sample.

Besides, the informational chapter <1227> - Validation of Neutralization Methods—Recovery comparisons of the USP 29 describes a test based on colony enumeration on the filter plated on agar compared to a positive control which has not been in contact with the product sample.

This test might give a more quantitative answer although microbial Colony Forming Units enumeration is subject to limitations in accuracy. This chapter also indicates that at least three validation runs have to be performed independently.

Membrane Filtration

As written in the USP and the EP, personnel performing sterility tests must use:

> Membrane filters having a nominal pore size not greater than 0.45 μm whose effectiveness to retain microorganisms has been demonstrated.

In general, any filter membrane named 0.45 μm should comply with the requirement as long as its retention efficiency has been demonstrated. Here, the technician working in the QC laboratory might already need some assistance to understand what's written between lines.

Publications describing retention mechanisms and ratings are widely available and could shed light of what is a true retention rating and what might need to be evaluated (Jornitz and Meltzer, 2001).

In order to summarize, 0.45 μm was the nominal pore size of sterilizing grade filters before the 1960s when Bowman et al. (1960, 1966) demonstrated that 0.45 μm filters failed to retain *Brevundimonas diminuta* formerly known as *P. diminuta*.

Questions have been often asked why 0.45 μm and not 0.2 μm which is the sterilizing grade retention rating of filter cartridges used in production? When raised most of the discussions drift to the experience level one has with 0.45 μm rated membranes. The advantage of this pore size over a 0.2 μm rated membrane is the enhanced ability of the growth media diffusion through the larger pore structure of the 0.45 μm membrane. Furthermore most of the sterility test membranes are highly adsorptive polymeric materials which capture the organisms not only by sieve retention, impaction, but also by adsorptive sequestration. So higher flow rate and better recovery might be obtained with 0.45 μm rated analytical filters without the loss of retentivity. Having said this, the debate over 0.45 versus 0.2 μm will never end. It is probably more a preference issue than a scientific exercise.

The more important issue is the proper validation of the sterility test membrane within the user's processes. This often does not include the retention capabilities of the membrane with the actual drug product. Often there is an exaggerated reliance on the manufacturer's data and certificates, which has been abolished in the sterilizing grade filter field as incomplete validation effort. It might be advantageous for the end user to obtain answers whether or not the retentivity of the sterility test membrane is truly given with the drug product tested. It well be that the drug product components influence the previously mentioned adsorptive capture (Jornitz and Meltzer, 2001). The retentivity efficiency and possible adverse influences of the product can be determined by the use of bacteria challenge tests. The 0.45 μm membrane could be challenged with specific test organisms inoculated in the drug product. If the drug product has an influence on the capture mechanism the organism might penetrate the sterility test membrane. Such possibility should be determined within the validation process. If not so done, how does one know what one captures or not.

Observation and Interpretation of Results

Both EP and USP specify that canisters containing media have to be incubated for 14 days. At intervals (see section on "*Incubation of the Canister and Growth Examination*") and at the end of the incubation period, the macroscopic evidence of microbial proliferation must be examined in both canisters.

When, after 14 days, both media do not present any evidence of microbial growth, the tested product complies with the test for sterility.

When growth is observed in the culture medium, the test may be considered invalid unless a fault, for example handling mistake, can be unequivocally demonstrated. Details are given in the section on "Investigation of positive results."

FDA Guidance for Industry – Sterile products Produced by Aseptic Processing Current Good Manufacturing Practice

This document from September 2004, while giving recommendations on aseptic processing operations also includes a chapter dedicated to sterility testing.

Environment

In the introduction, readers can find a very specific statement about the environment of the test which should compare to the environment used for aseptic processing operations. Mainly, based on historical reporting of false positive sterility testing, this statement also clearly highlights the use of isolators in order to minimize the risk of false positive results. It certainly is in the interest of the user to avoid false positive results, as such result would require extensive investigation and out-of-specification reports. Therefore, everything needs to be done to eliminate the potential for false positive tests, being a clean environment to training of the user. This topic will be addressed later in this chapter regarding trends in sterility testing.

Training and Qualification

An important recommendation is done about personnel qualification and training as part of a written program allowing regular update.

Companies providing sterility test units, systems and consumables are aware of this and provide training and qualifying sessions on a regular basis. Training and qualifying personnel can also be performed by internal resources. Actually, it has been often found that in a team of several technicians performing sterility tests in a quality control laboratory, there are some "experts" who are also responsible for maintaining a sufficient level of understanding of the application for others and especially new personnel.

As stated this issue is highly important to minimize the risks of sterility test failures and false positive results.

Sampling

Principally based on the statistical weakness of the test, due to the small size of the sample compared to the batch size, it is very important to test a representative sample.

Although a definition of a representative sample is given in the 21 CFR 210.3 (b) (21), the common practice matching the FDA Guidance for Industry's recommendation is to sample at the beginning, middle and end of manufacturing process. Besides, in case of unusual interventions on the production line during processing, samples should also be taken.

Investigation of Positive Results

When a positive result is found (growth in the culture media) an investigation is necessary in order to demonstrate whether the microbial growth comes from the product or lies in an error which generated a contamination during sampling or the sterility testing procedure. This event is commonly named a "false positive result".

Until the full investigation is conducted and accomplished, the lot is considered non sterile.

It must be mentioned here that it is extremely difficult to demonstrate the invalidity of the first result because the regulatory bodies hardly accept it. As a matter of fact, and although it is not clearly written in the regulations, this strictness finds its root in the increasing safety of the environment and disposable closed systems used for sterility testing.

The investigation should at least include:

1. *Microbial identification*: This helps to compare the identified strain(s) to the commonly found strain during environmental monitoring in the laboratory and the production environment.
2. *Review of laboratory tests and deviations*: This helps to define whether the laboratory regularly finds the identified microbe(s) in the laboratory monitoring. The analysis of deviations is essential and operators should stop the procedure before incubation if they think a deviation should have compromised the integrity of the test. It is common practice to perform multiple sampling in order to allow a second opportunity to perform the test again in this case.
3. *Investigation of process and process environment*: As the source of growth found in the culture media could find its root in the production itself or in the production environment, the complete aseptic process should be deeply analysed.

Besides, monitoring personnel, product presterilization bioburden, production record review and Manufacturing history should be deeply investigated.

It is then a very hard task to investigate a sterility positive creating a lot of additional work for the laboratory. Of course this favours the implementation of technical solutions to minimize the risk of false positives like isolators and closed disposable filtration devices.

PIC/S: PI 012-2 / 1 July 2004: Recommendation on Sterility Testing

The Pharmaceutical Inspection Convention and the Pharmaceutical Inspection Co-operation Scheme (PIC/S) are international instruments helping the cooperation between countries and authorities in the definition of Good Manufacturing Practice.

The PI 012-2 provides guidance for GMP inspectors to be used for training and preparation of inspections. It is also a very informative document for companies wanting to prepare for a GMP inspection of their sterility testing activities.

Among others, here are some aspects of sterility testing described in this document:

Training

In addition to what is found in the previously commented FDA Guidance for Industry (2.1) a special highlight is given in regards to the examination of the growth during and at the end of the incubation period. Actually, the growth of microbes is not always appearing in the same shape in a canister. For instance, growth sometimes occurs at the bottom of the canister where it is not easy to observe. Besides, as most of the time tests

are negative, laboratory technicians do not often see a microbial growth and need to be trained.

To mirror the need to qualify the operators in the previous FDA Guidance; the term of certification is used instead.

Environment

The sterility test should be performed in a class A laminar airflow cabinet located within a class B clean room, a class A clean room if available or in an isolator.

Validation

Chapter 11.6.2.4 of the PI 012-2 mirrors what is found in the international Pharmacopoeias but also recommends, as it is good practice, to re-validate every 12 months.

As this last recommendation is not a Pharmacopoeial requirement, re-validation of sterility testing is generally not repeated.

Chapter 11.6 clearly states that the positive controls (including validation) should be performed in a laboratory environment separate from the one where the product is tested. In other terms, it is not recommended at all to introduce microorganisms intentionally in the sterile test environment, even when it is part of a validation study. This seems obvious, but at least this recommendation makes sense, particularly when it is not available in many other official texts.

PIC/S: PE009-4 June 2006 Guide to Good Manufacturing Practice for Medicinal Products

The guide to GMP gives essential information related to manufacturing and some general indications in regards to sterility testing, although the head of quality control is considered in this document as key personnel.

Sampling

Page 57 of the guide (the chapter Quality Control) some indications are given about sampling:

As the guide do not only concerns aseptic filling, a recommendation for sampling of thermally sterilized products in their finale containers is also given: samples should be taken from the potentially coolest part of the load.

For products manufactured aseptically, the recommendations are similar to what is found in the FDA Guidance for Industry.

Environment

No specific recommendation is given related to the environment of sterility testing but the meaning of A, B, C and D classification is given and displayed in the following table:

	Recommended limits for microbial contamination			
Grade	Air sample CFU / m^3	Settle plates (diam. 90 mm) CFU/4h	Contact plates (diam. 55 mm) CFU/plate	Glove print 5 fingers CFU/glove
A	<1	<1	<1	<1
B	10	5	5	5
C	100	50	25	-
D	200	100	50	-



FIGURE 1 Sterility testing device. *Source*: Courtesy of Sartorius AG.

REALIZATION OF THE TEST ACCORDING TO REGULATIONS AND TROUBLESHOOTING

Introduction

Today, the method of choice for testing the sterility of sterile product by filtration is the disposable sterility testing device

A filtration device (Fig. 1) consist in two closed filtration canisters (Fig. 2) each containing a membrane filter and attached to a tubing allowing the filtration of products and incorporation of liquid culture media applied by a peristaltic pump (Fig. 3). At the end of the tubing set, an adaptor, commonly a collection needle, is attached in order to withdraw the liquid from the container in safe conditions. Depending on the container properties and design, several adaptors are available.

The product to be tested is filtered simultaneously on both membranes and culture media is introduced in each canister thanks to a set of preinstalled clamps. At the end of the procedure, one canister contains fluid thioglycollate medium and the other soybean-casein digest medium.

The tubing is cut and clamps are closed on top of the canisters to allow incubating in closed sterile conditions.

Pre-conditioning the Membranes

Reading the EP or USP, users should transfer a small quantity of a sterile diluent onto the membrane in the apparatus and filter. No clear reason is given in the pharmacopoeias to justify this step, as it is only written: “If appropriate”.

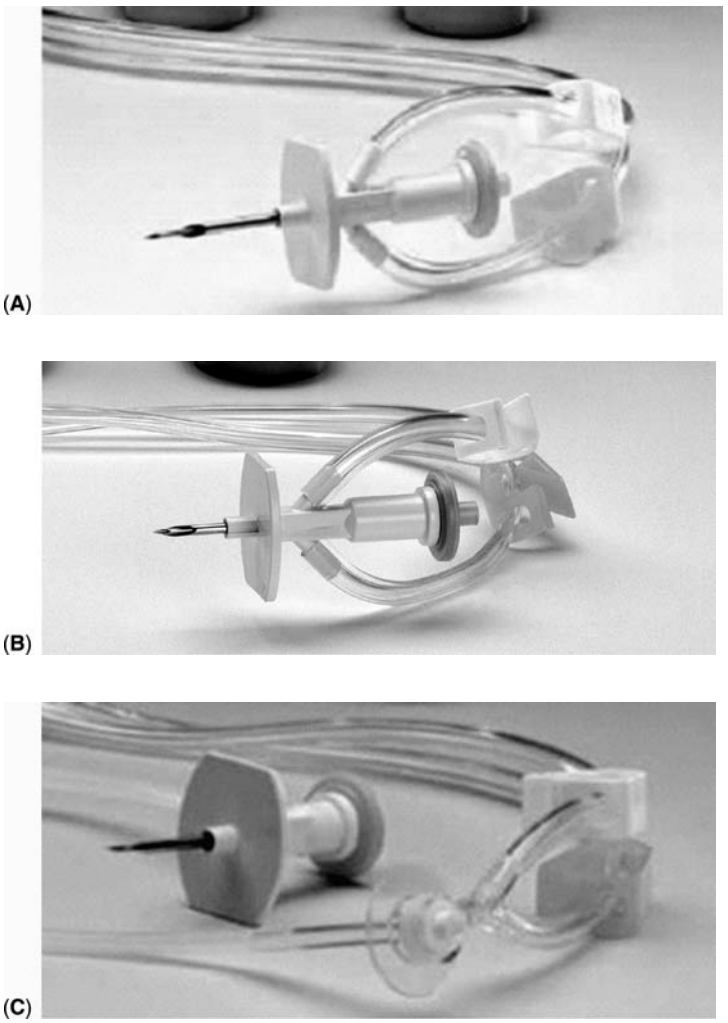


FIGURE 2 Adaptors for withdrawing liquid for sterility testing. (**A**) Adaptor for large volume parenterals in closed containers with integrated sterilizing grade filter. (**B**) Adaptor for small volume parenterals in closed containers with integrated sterilizing grade filter. (**C**) Adaptor for pre-filled syringes. *Source*: Courtesy of Sartorius AG.

Nevertheless, there are some explanations available and membrane pre-conditioning might be useful in one special case and for two main reasons.

First it must be mentioned that laboratories dealing with the test for sterility of antibiotics or products having inherent antimicrobial activity are used to condition the membrane filters before filtration.

The first reason is linked to the probability for liquids to find difficult to rinse voids in the canister. If such voids are likely to be present, and if no pre-conditioning of the membrane is applied, antimicrobial substances could hide there and would be very difficult to remove because not truly accessible to the liquid flow during the rinsing steps.

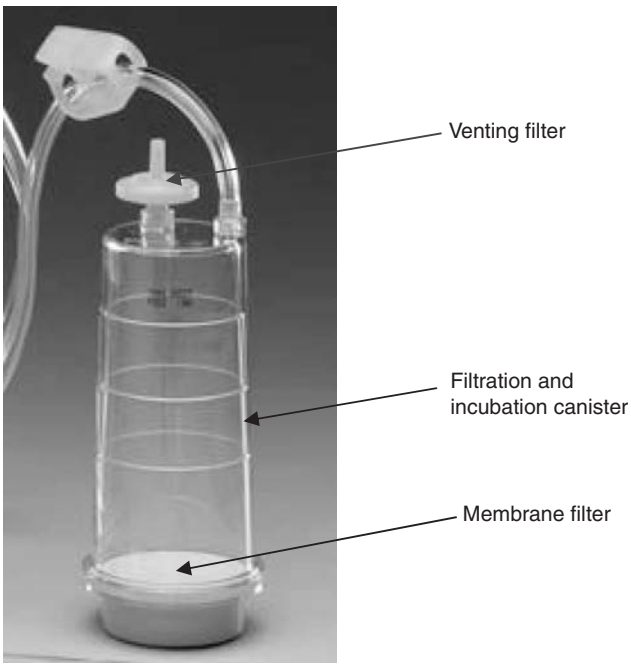


FIGURE 3 Sterility testing canister. *Source*: Courtesy of Sartorius AG.

The second reason is more related to chemistry than to physics.

Standard membranes generally made of mixed esters of cellulose contain strong polar groups and are a good support for hydrophobic interactions. These properties generate a non specific absorption capability that could help to bind antimicrobial substances, making them difficult or impossible to rinse. Of course in such a case, the risk of generating inhibition of microbial growth could be high enough to create false negative results.

Pre-conditioning of membranes, when done with fluid A according to USP or a neutral solution of meat or casein peptone at 1g/l according to EP, will create a protein layer as peptone proteins will bind to the active available groups of the filter material.

It will be then much more difficult for other substances to bind to the membrane later on.

General usage is 50 ml of fluid A according to USP or 50 ml of a neutral solution of meat or casein peptone at 1g/l according to EP.

Sample Filtration

The sample is then filtered immediately.

This is not mentioned in the regulations but the pump speed has to be adjusted to avoid product foaming and splashing in the canister.

As a matter of fact, product foaming could lead to a blockage of the filter vent creating pressure in the canister and damaging the integrity of the filter vent.

Product splashing, when having inherent antimicrobial properties could leave difficult to rinse drops that could be released in the culture media during incubation, generating a risk of false negative results.



FIGURE 4 Sterility testing peristaltic pump. *Source*: Courtesy of Sartorius AG.

The internal part of the canister is sometimes designed to guide the liquid along the wall, then limiting splashing and foaming.

When lyophilized antibiotics (powders) have to be filtered after dissolution, the users should make sure that all powder particles are dissolved. Undissolved particles could remain in the filter structure and be difficult to rinse, generating a risk of false negative results.

Membrane and Device Rinsing

When the product has antimicrobial properties, the membranes and devices must be rinsed not less than three times but not more than 5 times 200 ml. Generally users apply 3 times 100 ml of fluid A.

During the rinsing procedure, users must make sure that the complete portion of sample remaining on the canister surfaces and in the membrane will be rinsed. Generally, the diluent is introduced in the canisters while the vents are open to allow the introduction of a big amount of diluent in the canister.

Besides, in order to avoid any retention of antimicrobial substances in the sealing ring (Fig. 5) of the membrane, the design of the canister is of major importance.

Culture Media Introduction

After rinsing, the culture media are introduced separately in each canister and the tubes are cut on top of each canister.

Although the volume of culture media is not given in the EP or USP, the common practice is to use 100 mL. The volume of culture media is anyway part of the validation.

Incubation of the Canister and Growth Examination

The canisters are incubated for 14 days as mentioned in the regulations.

Thioglycollate media at 32.5 +/- 2.5˚C

Soybean casein digest at 22.5 +/- 2.5˚C

At intervals during the incubation users should examine the media for growth of microorganisms. The definition of intervals depends on the laboratory organization; if possible a daily observation is preferred. Examples of growth are shown in Figure 5.

A particular attention has to be paid concerning the thioglycollate media. This media contains agar to limit the diffusion of oxygen into it, in order to allow the growth of anaerobes. The fluid also contains a color indicator (Resazurin sodium) which turns pink when exposed to oxygen. Users have to make sure that at the end of the incubation period, not more than the half upper part of the culture media has turned pink. This would mean that the oxygenation is too high and might lead to the inhibition of anaerobes, generating a risk of false negative results. Besides, in fresh or stored media, not more than the upper third of the media should have turned pink. If so, the media must be restored by heating and cooled down promptly. Ready to use thioglycollate media in bottle is generally filled by the producer under inert gas (e.g., Natrium) in order to avoid adverse oxygen intake during transportation and storage.

Shaking the canister containing thioglycollate media is generally not recommended as it would unnecessarily oxygenate the media.

REVIEW OF WARNING LETTERS OF THE LAT YEARS

The following list of remarks and statements about failures related to sterility test were extracted from the FDA Warning Letters of the last 4 years when sterility test was

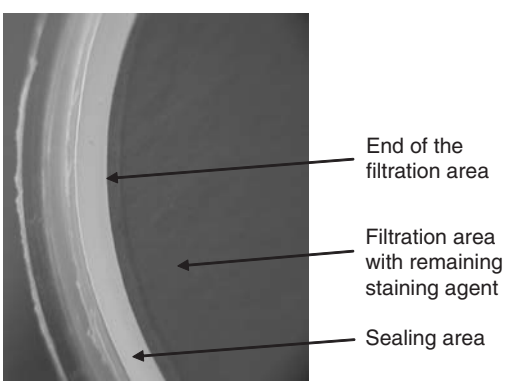


FIGURE 5 Membrane sealing zone. *Source*: Courtesy of Sartorius AG.

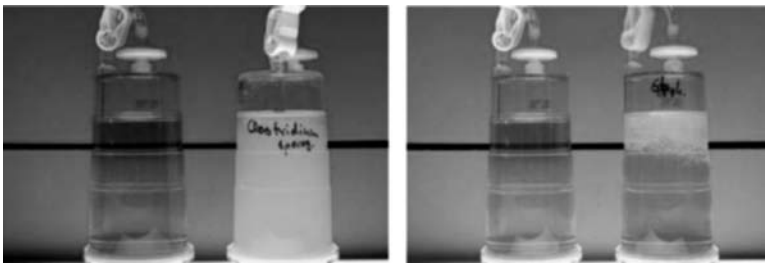
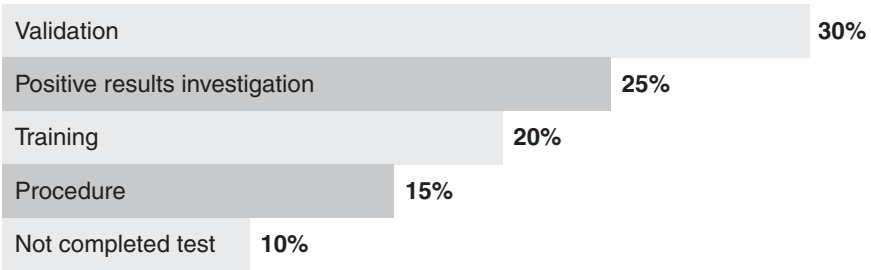


FIGURE 6 Examples of growth of *Clostridium sporogenes* (*left*) and *S. aureus* (*right*) after 2 days in thioglycollate media compared to negative control (left canister on each figure). *Source*: Courtesy of Sartorius AG.

mentioned. Such warning letters can serve as an indicator for the user to train the personnel or see what has to be focused on to eliminate any problems.

- insufficient training of the microbiologist technician,
- allowance of additional testing following a failed initial sterility test,
- improper gowning during sterility testing,
- absence of sterility test for a sterile product,
- failure to identify and discuss correlation of the sterility test isolate with microorganisms found in the facility environment or personnel,
- improper execution of the sterility test,
- failure of the analyst to follow the procedure,
- failure to complete sterility test,
- non adapted standard operating procedure (SOP) for the collection of sterility samples,
- incubation time of 7 days instead of 14,
- failure to test each batch for sterility,
- absence of sterility test in the preliminary study,
- failure to follow the sterility testing procedure,
- failure to correlate sterility test failures with incorrect filling parts,
- failure to undertake extended review of trends for sterility failures,
- failure to identify the type of errors or relate them to laboratory errors when having positive sterility tests,
- failure to demonstrate that the product does not inhibit the growth of microorganisms,
- absence of counts for the inoculated controls and samples,
- absence of satisfactory explanation of the procedure related to counts of microorganisms during validation,
- no assurance of the accuracy of assessments related to the identification of errors,
- lack of demonstration of not compromising package integrity during decontamination, leading to risks of false negative results,
- failure to follow manufacturer's requirements for storage of sterility testing culture media,
- failure to follow the SOP related to the investigation of positives,
- sterility test absent of the stability test program,
- failure to demonstrate absence of inhibition of residues leading to a risk of false negative result,
- bacteriostasis and fungistasis test not completed.

Importance of the following criteria in the analysed warning letters:



75% of the statements and remarks related to sterility testing directly or indirectly refers to Validation, Positive results investigations and Training. Whether there is a need of improvement of validation and regulatory requirements understanding is not demonstrated but likely to be. On the other hand proper training in handling and knowledge of regulatory needs, as suggested in nearly all regulations, could clearly help to get a better picture and reduce the number of apparitions of sterility testing remarks in FDA's warning letters.

TRENDS IN STERILITY TESTING

How to Minimize the Risks of False Positive and False Negative Results

Nowadays, most of the sterility tests are performed using a closed ready to use and disposable filtration set in laminar air flow cabinets or in isolators. However, there is still a part of the tests which is performed with reusable filtration systems mainly due to economical reasons. Using reusable sterility test equipment increases the risk of secondary contamination leading to potential difficult to prove false positive results.

Besides, the environment of the test for sterility of pharmaceutical sterile products is of major importance. Microbiological quality of the environment around the test must be controlled in a proper way and is according to regulations classified as a class A (ISO 5) laminar air flow (LAF) cabinet within a class B (ISO 7), an isolator or a class A (ISO 5) clean room when available.

This means that the quality required is closed to sterility.

Maintaining this quality is essential for minimizing the risks of introducing microorganisms from the environment into the canisters.

As the retest of a product batch is extremely problematic, users need more and more to improve the quality of the environment targeting the lowest risk of obtaining false positive results. This is why class A LAF must be within a class B environment and why isolators are constantly gaining interest for the sterility test application.

More, recently the FDA clearly stated in its "FDA Guidance for Industry – Sterile Products Produced by Aseptic Processing Current Good Manufacturing Practice" that: "the use of isolators for sterility testing minimizes the chance of a false positive test result."

As a matter of fact, the isolator technology allows personnel to work outside the critical area by using gloves and sleeves or half suits. It is well known that the major source or microorganism in sterility testing laboratory is personnel; any mean providing a physical separation between the working place and the analyst will with no doubt limit the risk of microorganism release at the critical point.



FIGURE 7 La Calhène isolator with soft walls and transfer isolator.

There are many isolator suppliers on the market and several technologies can be found.

One of them is the soft wall isolator (Fig. 7) equipped with one or several half suits or with gloves and allowing the introduction of material and consumables through transfer ports connecting the work station to transfer isolators used for gas decontamination.

The same isolation concept can also be found with rigid walls.

This concept allows some flexibility for transfer operations and a reduced frequency of gas decontamination of the work station.

Another concept consists of a work station which can be fully opened, generally in front of it, but not using transfer isolators connected to the work station (Fig. 8).

In this case, the main work station must be decontaminated after each loading of material and samples required for the test.

Although the advantages of isolation technology are undeniable, it generates some new challenges for quality control personnel.

One of them lies in the use of chemical agents for the decontamination of material and consumables. Generally, isolators are decontaminated with vapor phase hydrogen peroxide or a mixture of hydrogen peroxide and peracetic acid.

Packaging material, culture media bottles, and other consumables and reagents which are necessary for performing the test are in direct contact with the chemicals during the decontamination process. In case the chemicals penetrate inside the packaging, this could have an adverse effect on the growth of microorganisms, potentially generating a risk of inhibition, giving false negative results. In the USP 29 chapter <1208> Package Integrity Verification the risk of penetration of chemicals used for decontamination of isolators inside canisters used for sterility testing is clearly specified.



FIGURE 8 SKAN rigid wall isolator.

Besides, the "FDA Guidance for Industry – Sterile Products Produced by Aseptic Processing Current Good Manufacturing Practice", states that users should validate that there is no risk of false negatives.

To minimize this risk, the packaging material should be adapted and technical solutions are already available. The trend is to get consumables packed in multilayer plastic packaging or multilayer plastic and aluminum packaging.

Use of Alternative Methods

In recent reports, new alternative non-compendial methods have been used for testing the sterility of sterile products.

For instance, in February 2004 the American company Genzyme got the FDA approval for the use of the BactAlert system from Biomérieux as an alternative sterility test using the ability of microbes to produce CO_2.

This allows obtaining the results very early compared to the compendial 14 days. In this special case, the sterile product has a very short shelf life of a few days only. It is then impossible to apply the traditional method with last 14 days. Besides, because the product is a cellular product, it creates turbidity in the culture media which renders the sterility test even more difficult to perform in the compendial way.

More recently, another case has been reported where Alcon, U.S.A. got the FDA approval for the use of the Chemscan RDI system from AES Chemunex based on the labelling of viable microorganisms with a fluorescent substrate.

The introduction of alternative microbiology methods for sterility testing is so far very limited to some specific cases, essentially in the U.S.A. and it is not really clear how long the compendial traditional way will remain the preferred method. However, the new technologies have gained an increased interest in the last years and even found the ways of FDA approvals in a supposed very conservative environment.

The use of alternative methods for sterility testing in replacement of the compendial method has not shown up in Europe either.

CONCLUSION

Sterility testing is still considered a critical analytical step for drug product release. Since it is as critical and manifested in the release criteria of a batch any false positive result can be extremely disturbing, especially as full scale investigation are lengthy and would delay the release of a batch. In instances such investigation can be inconclusive which would jeopardize such batch.

For this reason it is of importance to utilize best practices to avoid any potential cross contaminant. New disposable device technologies to perform sterility tests with a lower risk of a false positive result, as well as isolator technology which creates an even cleaner environment support such practice. Certainly new equipment technologies enhanced the accuracy of the sterility test, still the well established membrane sterility test method is lengthy. New rapid microbial detection technologies will be developed and approved.

In any case, the accuracy of a sterility test is only as good as the responsible analytical personnel performing such test. Training should never be forgotten, as the equipment is only be as good as the user of such.

This chapter described the common regulatory practices, but also detailed what is required to perform an appropriate sterility test and interpret such test. Equipment

designs, especially in regard to disposable canisters are essential in critical application like the sterility testing of bacterical or bacteriostatic solutions.

Other topics, not yet concluded were also described. One of such being the validation of the sterility test membrane. So far there is no challenge standard to verify that the pore size rating quoted has the reduction value required. However, such challenge test verification would be supportive for any validation process.

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26

Bacterial Biofilms in Pharmaceutical Water Systems

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INTRODUCTION

Bacterial contamination of purified waters continues to present a challenge to pharmaceutical, medical device, and cosmetics manufacturers. Biofilms are ubiquitous other types of industrial water systems, and create similar health-related and economic problems (Kulakov et al., 2002). The presence of bacteria and their associated endotoxins (bacterial pyrogens) in pharmaceutical waters poses the single greatest threat to product quality. Regulatory recalls directly related to bacterial bioburden are often associated with contamination of purified water and water for injection; a review of the impact has been published by Mittelman (Mittelman, 1995).

While the abiological particle loading of a given system usually remains relatively constant, small changes in environmental conditions can drastically influence the number and type of microorganisms present. Regardless of the prevailing conditions, however, the majority of bacteria in pharmaceutical water systems are associated with surfaces as biofilms. Biofilms may be described as surface accretions of bacteria, extracellular polymeric substances (slime), and entrained organic and inorganic detrital material. Desorption of bacteria and bacterial aggregates from biofilms gives rise to the majority of bulk-phase, or planktonic, bacteria in fluid-handling systems.

Although microporous membrane filters are designed to reduce or eliminate planktonic bacterial numbers, it is important to recognize that filtration efficacy and effective filtration area like are inextricably linked to bacterial biofilms associated with various surfaces. The high surface area/volume ratios associated with the many components of a purified water system create considerable niches for biofilms and, by extension, generate water system bioburden. It is this bioburden generated at surfaces that is most often responsible for transmembrane pressure increases resulting from fouling of microporous membrane feedwater surfaces.

THE MICROBIAL ECOLOGY OF PURIFIED WATERS

Virtually all bacteria isolated from purified water systems are Gram-negative, non-fermentative bacilli. The Gram-negative cell wall consists of multiple layers of

phospholipids surrounded by a lipopolysaccharide structure. This multilaminate structure may afford the cell protection from the extremely hypotonic environment that is intrinsic to purified water systems. These bacteria are heterotrophic, requiring the presence of reduced organic compounds as energy sources. Therefore, these compounds serve as limiting growth factors in purified water systems. The term "oligotroph" has been assigned to organisms that are capable of growth in media containing < 1 mg/L organic carbon (Ishida and Kadota, 1981). In general, a positive correlation exists between assimilable organic carbon levels and planktonic bacterial numbers in purified waters.

The International Space Station has a number of "purified" water systems associated with both spacecraft operations and crew usage. The types of microorganisms and their distribution on surfaces are very similar to those found in pharmaceutical water systems (Novikova et al., 2006). In addition to the concerns of bioburden and endotoxins as a direct health threat, microbially influenced corrosion is also of concern (Roman et al., 2001). "Ultrapure" water systems found in the semiconductor (Kim et al., 1997; Kim et al., 2000) and nuclear power (Chicote et al., 2004; Sarro et al., 2005) industries are also susceptible to bioburden contamination resulting from biofilm development. Again, the types and distributions of microbial populations are similar in these various water systems.

Population densities in distilled water have been reported to exceed 10^6 colony-forming units per milliliter (CFU/mL)(Carson et al., 1973). McFeters et al. (McFeters et al., 1993) found 10^2–10^3 CFU/mL in a model laboratory water system, with many of the same system components (deionization beds, UV, and microporous membrane filters) that are seen in pharmaceutical grade purified water systems. Like a number of other workers (Mittelman et al., 1987; Patterson et al., 1991; Martyak et al., 1993), this group found that significantly grater numbers of bacteria (ranging up to 10^5 CFU/cm^2) were associated with various surfaces within the water production and distribution systems.

Starvation and Survival in Purified Waters

When one of more essential growth factors are limited, as is often the case in benthic environments and purified water systems, bacteria use a number of strategies designed to ensure their survival. In the short term, "starved" bacterial tend to use endogenous energy reserves for replication; therefore, one strategy for survival involves replication to increase the probability of species survival once additional energy sources become available (Novitsky and Morita, 1978). In marine systems, limited in assimilable nutrients, some bacteria reduce their cell volume, forming "ultramicrobacteria." Tabor et al. (1981) demonstrated that these organism, many of which were < 0.3 μm in diameter, were capable of passing through 0.45 μm pore microporous membrane filters. Their findings tend to corroborate the work of Christian and Meltzer, 1986 and others regarding bacterial penetration of "sterilizing grade" microporous membrane filters.

A number of investigators have described bacterial growth and biofilm formation in "ultrapure" water (18 MΩ cm; < 10 μg/L TOC). Kim et al. (2000) described bacteria with a wide range of assimilable organic carbon utilization capabilities in semiconductor waters. Similar types of bacteria were recovered from "ultrapure" water (Chicote et al., 2005), and from biofilms (Chicote, et al. 2004; Sarro, et al. 2005) in spent nuclear fuel pools.

Objectionable Organisms

The U.S. Pharmacopeia not only stipulates the maximum action limits for purified water and other pharmaceutical waters, but also specifies that it not contain objectionable organisms. Included in this group are coliform bacteria, which by compendial requirement cannot exceed limits promulgated by the U.S. EPA. Currently, a limit of < 1 CFU/100 mL total coliforms is in place. In addition, it is important to consider that changes in the microbial ecology of both the feedwater and the manufacturing environment may occur at any time. The significance of organisms in nonsterile drug forms requires an evaluation with respect to the product's intended use, the nature of the preparations, and potential hazard to the user.

Increasingly, however, noncoliform, heterotrophic bacteria previously considered "normal inhabitants" of purified waters are considered objectionable. Indeed, the prevailing view among manufacturers and the FDA is that objectionable organisms (other than the stipulated prohibition against coliform bacteria) are product application-defined. For example, purified water used in the manufacture of non-sterile ophthalmic preparations should be free of *Pseudomonas* spp. in particular, *P. cepacia* and *P. aeruginosa*. The definition of "objectionable organisms" in pharmaceutical waters is likely to broaden in the future: As new products and product applications are introduced, it follows that a number of existing and emerging environmental bacteria will be proscribed.

With an increasing recognition of the role of bacterial biofilms plays in the generation of water systems bioburden, it is likely that limits will eventually be placed on both "normal inhabitants" and "objectionable organisms" associated with surfaces. Current limitations associated with sampling and enumeration techniques for this significant subpopulation of water systems bioburden prevent the establishment of concern and action limits. Biofilms have been implicated as a reservoir for pathogenic bacteria in drinking water distribution conduits (Wireman et al., 1993; Rogers et al., 1994), hospital water systems (Alary and Joly, 1992), hemodialysis water systems (Phillips et al., 1994), and dental treatment units (Schulze-Robbecke et al., 1995; Shearer, 1996). However, as McFeters et al. (1993) noted, no comprehensive studies on the microbial ecology of pharmaceutical grade water systems–biofilm communities in particular–have been carried out.

The specific physicochemical environment associated with purified water systems is the primary determinant of the microbial ecology. As was previously noted, similar types of bacteria are found in purified water systems associated with the pharmaceutical, dialysis, semiconductor, and aerospace industries (Kim et al., 2000; Chicote et al. 2005). Unless these waters are maintained sterile—free of any viable microorganisms—it is not possible to exclude bacteria that are adapted to life in an extremely oligotrophic environment. Many of these organisms include *Pseudomonas* spp. that are of concern to the pharmaceutical industry.

GENESIS OF BACTERIAL BIOFILMS

Perhaps the most significant adaptive mechanism used by nutrient-limited purified water bacteria involves adhesion to surfaces. Indeed, the majority of bacteria in a nutrient limited environment, such as an ultrapure water system, are attached to surfaces (Fig. 1). Surfaces afford these organisms three major advantages over the bulk phase:

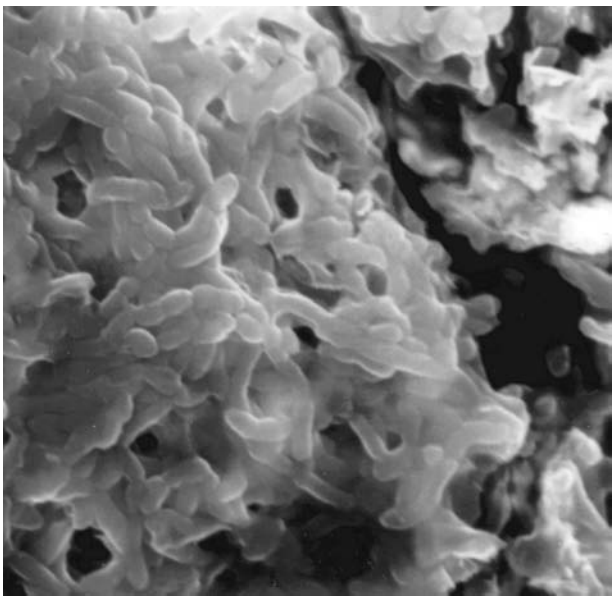


FIGURE 1 Scanning electron micrograph of a water system biofilm on 316 stainless steel.

1. Trace organics, which can serve as nutrient sources, concentrate on clean surfaces shortly after their immersion.
2. Surface associated bacteria tend to produce extracellular polymeric substances (EPS), which can further concentrate trance growth factors.
3. Bacteria in biofilms are afforded some protection from antagonistic agents such as biocides, heat treatments, and other inhibitory factors.

Several workers have shown that nutrient-limiting environments promote the attachment of bacteria to surfaces (Mittelman et al., 1987; Marshall, 1988). The relatively high surface area/volume ratio associated with industrial water systems provides ample space for bacterial attachment. Any one of the many systems used in the purification of feedwaters is therefore a potential reservoir for contamination. In terms of biological generation, however, granular activated carbon columns (Johnston and Burt, 1976; Collentro, 1986), reverse osmosis (RO) and ultrafiltration membranes (Ridgway et al., 1985; Laurence and Lapierre, 1995), ion-exchange (DI) systems (Flemming, 1987), RO/DI water storage tanks (Collentro, 1996), and microporous membrane filters (Collentro, 1996), are the most significant areas of concern. Many of the same concerns surrounding biological contaminants in pharmaceutical waters have been reported in other industrial fluid-handling systems (Mittelman and Geesey, 1987; Mittelman, 1991).

Biofilm Development

Development of bacterial biofilms in aqueous environmental proceeds in a three-step process: (*i*) accretion of trace organics on the surface, (*ii*) primary ("reversible") adhesion, and (*iii*) surface colonization (growth of adherent bacteria). Bacteria form microcolonies on surfaces that may or may not be continuous in their distribution, as illustrated in Figure 2. Although biofilms are known to be the source of bulk phase or

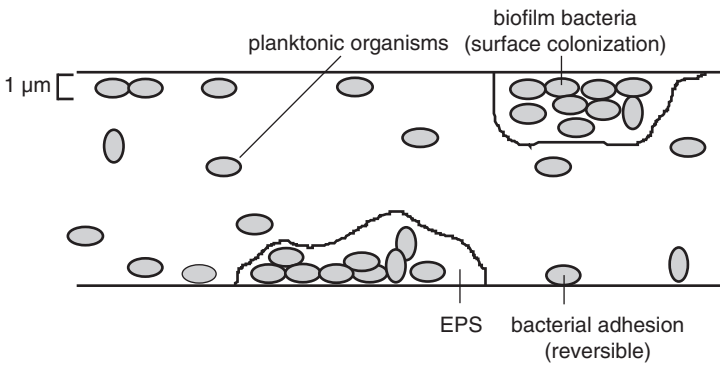


FIGURE 2 Genesis of bulk-phase and biofilm bacteria in a pipeline.

planktonic bacteria in a water system, there is no discrete relationship known between the relative population sizes. This is due to the stochastic nature of adhesion, colonization, and desorption–all of which are affected by a number of interrelated physiochemical factors (Table 1).

The elaboration of EPS by reversibly bound bacteria stabilizes the initial adhesion event. Extracellular polymeric substances often carry a strong net negative charge, which may be important in the stabilization of succeeding adherent organisms. Significantly, it is the presence of EPS that affords bacteria in pharmaceutical water systems protection

TABLE 1 Bacterial Adsorption to Surfaces in Purified Waters

Adhesion event	Mechanisms	Effect on adhesion	Key variables
Reversible phase	Balance between double layer repulsion and van der Waals attractive forces	Cells are maintained a finite distance from the surface; cells are loosely held and easily removed by mild hydraulic forces	pH; ionic strength; organic carbon concentration; fluid hydraulics
Irreversible phase	Adhesion is mediated by bacterial cell surface structures such as extracellular polymeric substances; hydrophobic actions exist	Cells are firmly adhered to surfaces; removal is effected by mechanical abrasion or alterations in the nature of adhesion factors	Electrostatic interactions; surface free energetics; temperature; hydrophobic interactions
Desorption	A stochastic event that may be dependent on cell-cell communication and surface density; bulk-phase physicochemical factors may be important	Fluid hydraulic forces exceed adhesive forces; bacteria and/or their biomass constituents detach from the surface; desorbed bacteria can colonize downstream surfaces	Fluid hydraulics temperature; ionic strength; surface tension; cell-cell signaling (quorum sensing)

from chemical treatments and, possibly, flowing steam/hot water treatment. EPS can constitute a significant fraction of the total surface biomass (bioburden) as is shown in Figure 3.

Biofilm formation is also controlled, in part, by quorum sensing, a bacterium-bacterium communication mechanism that is related to population density. (Costerton et al., 2005; Fux et al., 2005). Inhibiting precursor molecules involved with this mechanism may prevent/disrupt bacterial biofilm formation. However, to-date, the applications for disruptive treatments of this nature have been limited to the clinical environment; for example, device-related infections.

Endotoxins present in pharmaceutical water systems—like planktonic bacteria—originate with bacterial biofilms. Rioufol et al. (1999) found >1000 endotoxin units/cm^2 of surface were associated with a medical device biofilm. Endotoxin is present in both free and bound forms, and is continually sloughed-off from growing Gram negative bacteria. Removal of biofilms from surfaces also results in the elimination of most endotoxins from the bulk phase water (Shinoda, 2004).

Influence of Surface Characteristics

The influence of surface roughness and surface energetics on the development of bacterial biofilms has been examined by a number of investigators. Vanhaecke and Haesevelde (1991) and Vanhaecke et al. (1990) reported that surface roughness can have an influence on the adhesion kinetics of some bacteria. They suggest that hydrophobic strains of *P. aeruginosa* show greater adhesion to electropolished stainless steel than strains possessing more hydrophilic cell surfaces. Indeed, the kinetics of adhesion for the former strain types were similar on both polished and electropolished stainless steels. Other investigators reported significantly different "adhesion kinetics" for the same organisms (Pedersen, 1990; Quirynen et al., 1993; Verheyen et al., 1993). Conversely, Arnold and Suzuki (2003) suggested that bacterial adhesion to electropolished stainless

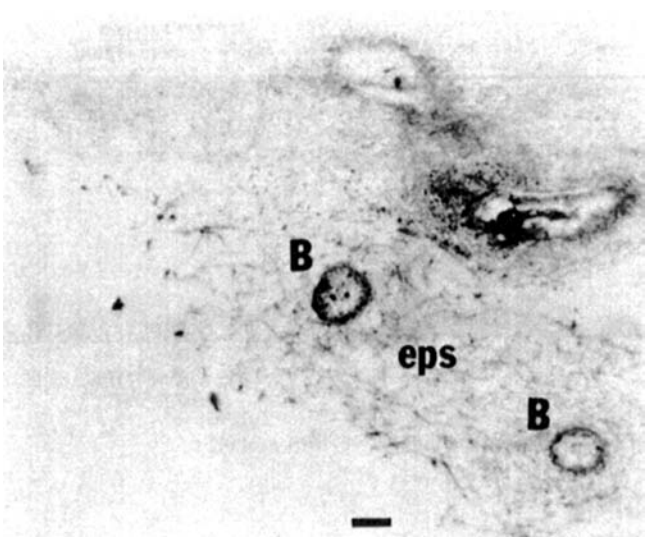


FIGURE 3 Transmission electron micrograph of a bacterial biofilm section adjacent to a 316 stainless steel surface. B = bacterial cell; eps = extracellular polymers. Bar = 1 μm.

steel surfaces is significantly reduced relative to "rougher" steels. Riedewald (2006) has evaluated the literature concerning the influence of surface roughness on bacterial adhesion: he concludes that there is no real benefit to specifying highly polished (i.e., smooth) surfaces if the desired outcome is reduced bacterial attachment. In particular, he notes that "the roughness of stainless steel surfaces in the range of 0.01–3.3 μm Ra has no significant influence in cleanability."

There are no standardized methods for measuring bacterial adhesion under conditions designed to simulate those of the processing environment. Evaluations of surface effects on adhesion are very dependent on the methodologies employed. Evaluations are further complicated by the observation that adhesion kinetics are significantly influenced by a number of interrelated environmental and surface factors, some of which are listed in Table 2.

It is apparent that surface characteristics are important during the initial stages of adhesion (Marshall et al., 1971; Dahlback et al., 1981). It is unlikely, however, that surface structure or free energy considerations play important roles following the initial adhesion event. Following the initial adhesion event, succeeding bacteria may not contact the underlying surface. Similar findings have been reported for fluid hydraulic effects. Characklis (1990) showed that fluid hydraulics can affect the initial rate of colonization; however, following this initial "lag" phase, biofilm development appears to proceed somewhat independently of fluid flow.

TABLE 2 Environmental Effects on Bacterial Adhesion Parameters

Bulk-phase environmental parameters	Effect(s) on bacterial adhesion in aqueous environments	Conditions minimizing adhesion[a]	References
pH	Cell surface charge; adhesion to surfaces; binding capacity of cell surface; extracellular polymer stability	<6	Herald and Zottola, 1987; Characklis, 1990; Richards et al., 1993
Temperature	Metabolic activity; heat-shock protein analysis	$>40^{\circ}$C to minimize viscosity	Hoadley, 1981; Aranha and Meeker, 1995
Ionic strength/ osmolarity	Cell surface charge; adhesion to surfaces	Greater ionic strength increases bacterial cell surface negative charge densities	Baldwin et al., 1988; Fletcher, 1988; Geertsema-Doornbusch et al., 1993; Baldwin et al., 1995; Baldwin et al., 1995
Assimilable carbon	Metabolic activity; organism size; cell surface hydrophobicity	Decreasing carbon and starvation result in shrinking cell size and increases in cell surface hydrophobicity	Novitsky, et al., 1978; Dahlback et al., 1981; Morita, 1982; Morita, 1985; Nystrom et al., 1992

[a] Surface- and organism-dependent.

Flow Effects

In the section on "Influence of Surface Characteristics," the effects of surface roughness, Ra, on microbial adhesion and biofilm development were discussed. As with surface roughness, there is much debate surrounding the influence of flow on the formation and stability of biofilms. The often-stated "recommended" minimum flow rate of 5–7 feet/s does not appear to have any scientific support, and is based on a "standard" 2 inch diameter pipe (Meltzer and Jornitz, 2006). Indeed, most of the peer-reviewed literature suggests that flow rates have a minimal effect on biofilm development and stability. Neither biofilm development or planktonic cell numbers were significantly influenced by purified water flowing at 1.5–5.2 m/s and the corresponding shear stresses of 9.1–84 N/m^2 (Soini et al., 2002; Rickard et al., 2004; Tsai et al., 2004; Ramasami and Zhang, 2005; Rupp et al., 2005). There is also evidence to suggest that "stronger" biofilms may form at higher shear forces, up to a critical shear force (Liu and Tay, 2001).

Given the submicron size range of bacterial footprints on surfaces, which place attached cells within a viscous fluid sublayer under most flow regimes, it is unlikely that maintenance of a high flow rate alone would retard adhesion and biofilm development. There may be a benefit to increased flow rates where UV, microporous membrane filtration, or other planktonic bacterial treatments are employed. In these cases, higher flow rates would increase the frequency of interaction between planktonic cells and the treatment device.

BIOFILM DETECTION

Since biofilm associated bacteria are not detected by current test methodologies, serious underestimates of contaminating populations result. In addition, this inability to detect the major source of bulk-phase biological contaminants can result in an overestimate of treatment efficacies. Biofilm bacteria can be quantitatively recovered and enumerated from surfaces by using classical cultural techniques (Martyak et al., 1993; Patterson et al., 1991). Pipe surfaces may be swabbed with sterile cotton applicators, then plated onto the appropriate medium (R2A, etc.). However, the difficulty in reproducibly sampling surfaces has precluded more widespread testing of pharmaceutical systems. Currently, there are no commercially available biofilm samplers available for the pharmaceutical industry. Sacrificial pipe sections, including a modified Robbins device, have been used for recovery of biofilm bacteria in purified water systems (Mittelman, et al., 1987; Martyak, et al., 1993). However, these types of sampling systems and the associated analytical techniques are somewhat cumbersome and time-consuming.

Techniques for recovering biofilm bacteria from surfaces usually involve mechanical disruption through physical scraping or sonication. These techniques result, at best, in semiquantitative assessments of biofilm population sizes. This is due in part to the small size of bacteria, which often are smaller than the surface Ra. In addition, many biofilms are tightly adhered to surfaces and effective removal of cells (e.g., via sonication and surfactant application) may kill otherwise viable organisms. Although the techniques for removal and enumeration of biofilm bacteria are evolving, a number of methods have been described (Mittelman, 1998; McFeters et al., 1999; Mittelman, 1999; Haberer and Mittelman, 2003; Marion-Ferey et al., 2003).

In any process, it is important to monitor possible contamination so corrective action can be initiated as rapidly as possible. The ideal monitors should be nondestructive so they will not inhibit or damage the biofilm. If the test systems can be placed in a supply lines or in the system ahead of purification/disinfection systems, then treatments

can be modified to maintain microbiological control. A number of emerging on-line detection systems have previously been reviewed (Mittelman et al., 1993; Mittelman, 1994; Mittelman, 1998; Mittelman, 1999; Flemming, 2003; Haberer, et al., 2003); three technologies with potential applications for the pharmaceutical industry are described herein.

Fourier Transform Infrared Spectrometry

Fourier transform infrared (FTIR) spectrometry operated in the attenuated total reflectance mode allows the detection of bacterial biofilms as they form on a crystal of zinc selenide or germanium (Nichols et al., 1985; Schmitt and Flemming, 1998). The amide stretching of the proteins and ether stretch of the carbohydrates are clearly detectable when bacteria attach to surfaces. Often the nutrients attracted to the surface from the bulk phase are also clearly indicated by their infrared fingerprint. If the water system contained IR "windows," biofilm formation could be monitored and an indication of the chemical nature of the contamination would be apparent. Periodically, some mechanism for cleaning the "window" would have to be provided. A detection limit of $\approx 5 \times 10^5$ *Caulobacter crescentus* cells per square centimeter was obtained using a germanium substratum monitored in a ATR flow cell (Nivens et al., 1993). This non-destructive technique holds promise as an online monitoring tool for bacterial colonization compounds in purified water systems. The ability to monitor biomass "signature compounds" may be of particular utility in assessing chemical treatment efficacies against purified water system foulants.

Fluorimetry

The use of fluorimetry for on-line monitoring of biofilm development has been described for monitoring of antifouling (Mittelman, et al., 1993) and uncoated stainless steel surfaces (Khoury et al., 1992). Reduced NAD compounds along with the aromatic amino acids (e.g., tyrosine and tryptophane) can be monitored as indicators of cell energy charge and biomass, respectively. As with FTIR spectrometry and quartz crystal microgravimetry (described below), this technique holds promise for application to monitoring purified water system fouling. Detection limits remain relatively poor, however, in the range of $\approx 10^5$ cells/cm^2.

Costanzo et al. (2002) have described the application of the Scan RDI system for detecting bacteria and other microorganisms on membrane surfaces. Individual cells or aggregated cells can be detected at a resolution of approximately 0.1 μm. Fluorimetry has also been applied to the detection of specific clinical and environmental bacteria, some of which also have significance for biodefense applications (Chuang et al., 2001; Ji et al., 2004).

Quartz Crystal Microbalance

The presence of material of a significantly different intrinsic viscosity than that of the water can be detected as a change in frequency of a vibrating quartz crystal in quartz crystal microbalance technology. The surface can also be used as an electrode to produce both the microbalance and electrochemical signals (Deakin and Buttry, 1989). This technique was applied to monitoring biofilm formation in a simulated purified water system for the space station project. Detection limits of $\approx 10^5$ cells/cm^2 were obtained using an AT-cut quartz crystal (Nivens et al., 1993).

TREATMENT

Inactivation and removal of bulk-phase bacteria following the most commonly employed physical and chemical treatments is usually complete. Within days or weeks of treatment, however, bulk-phase bacterial levels are often as high or higher than pretreatment levels. Bacteria existing in sessile biofilms are protected from the effects of antimicrobial agents and, to some extent, physical treatments (e.g., steam, hot water). This resistance to inactivation is very different from that associated with classical antibiotic resistance, which involves specific mechanisms of resistance that are often unique to individual microbial species (Costerton, et al., 2005; Fux, et al., 2005). A number of studies have shown that repopulation of purified and other industrial water systems is mediated by the presence of adherent bacterial populations (Mittelman, et al., 1987). The key to effective treatments, therefore, is inactivation and removal of this population of contaminating biological particulates.

Any treatment employed must be compatible with water system materials of construction. For example, although application of sodium hypochlorite can be a very effective means of inactivating and removing biofilm, this chemical can cause chloride-induced stress cracking of stainless steels (Meltzer and Jornitz, 2006). Understanding component compatibility is also essential to the development of an effective equipment cleaning program.

Physical Treatments

Empirical experience has demonstrated the efficacy of frequent flowing steam or hot water treatments of pharmaceutical water systems (Agalloco, 1990) and in other industrial water systems (Pflug et al., 2001). Most vegetative bacteria (non-spore forming) are killed at temperatures between 50 and 65°C (Demain and Solomon, 1986). As with any type of antimicrobial treatment, the two key parameters are contact time and concentration (or temperature). Although there have been no reports of heat-resistant populations of bacteria in pharmaceutical water systems, problems with heat inactivation have been described for *Legionella* spp in distribution systems for porable water (Stout et al., 1986) and *Listeria monocytogenes* in dairy products (Lee and Frank, 1990).

While both flowing steam and hot water (80°C) appear to be effective at inactivating biofilm bacteria in pharmaceutical waters, neither treatment will effectively remove the biomass from surfaces, including EPS and constituent endotoxins. However, it is clear that combining heat treatment with the appropriate chemical treatment results in a synergistic effect in terms of bioburden reduction (Holmes et al., 2004). Depyrogenation of surfaces cannot be reliably accomplished using hot water or steam-in-place treatments. Bacterial lipopolysaccharides are notoriously difficult to remove from surfaces (Novitsky, 1984; Ludwig and Avis, 1990). A significant number of problems with pyrogens have been reported in hemodialysis facilities with improperly treated purified water systems (Murphy et al., 1987; Laurence, et al., 1995). Bacterial biofilms within various components of these systems are likely the major source of endotoxins (Vincent et al., 1989).

Ultraviolet irradiation at 185 or 254 nm is an effective process for inactivating planktonic microorganisms in pharmaceutical and other industrial waters (McAlister et al., 2002; Anderson et al., 2003). However, UV has not been shown to be effective against attached bacteria in biofilms. In part, this is due to the poor penetration of UV, either at 185 or 254 nm, with the energy available for cell interaction

decreasing by the square of the distance from the UV source. Meltzer (Meltzer, 1997) has described UV applications for pharmaceutical water systems.

Chemical Treatments

The development of process- and product-compatible biofouling treatments has long evaded manufacturers of critical products that require purified water as a raw material. Of all the physical and biocidal treatments applied to pharmaceutical grade purified water systems, ozone appears to hold the greatest promise as an effective product compatible biocide. With the development of fluoropolymer-based microporous membranes for pharmaceutical applications, ozone can be applied throughout a USP purified or water-for-injection system. The technology is currently available for inactivation of residual ozone levels in situ via UV irradiation (Meltzer, 1993). Most of the problems with ozone application to purified water systems have been in the realm of component compatibility. Since ozone is an extremely reactive oxidant, its use is confined to systems constructed of such inert materials as Teflon and other fluoropolymers.

Hydrogen peroxide, a commonly employed purified water system treatment chemical, can also be readily degraded by ultraviolet light. However, it has relatively little activity against attached bacteria in biofilms. There is also evidence that suggests that hydrogen peroxide requires catalytic concentrations of Fe^{2+}, Ni^{2+}, or Cu^{2+} for optimal biocidal efficacy (Block, 2001).

Surfactants such as the quaternary ammonium compounds have excellent antimicrobial activity in addition to their intrinsic detergency. Synergistic combinations of chlorinated or brominated compounds with surfactants may provide additional activity against bacterial biofilm populations. Their ability to interact with surfaces does, however, create problems related to removal of these compounds following application. Larger volumes of rinse water are required for the surfactant compounds than, for example, for hydrogen peroxide.

Peracetic acid, 0.02% (v/v), has been found to be an effective treatment agent for both deionization resins and associated water system components (Flemming, 1984; Alasri et al., 1993). This compound is sometimes used in synergistic combinations with hydrogen peroxide and/or UV irradiation. Along with its true biocidal properties (i.e., sporocidal activity), peracetic acid is an effective depyrogenating agent and is used extensively in the hemodialysis industry, both for water system disinfection and for ultrafilter sterilization/depyrogenation.

More recently, citric acid has been evaluated for its ability to inactivate and remove biofilms from water distribution systems. Tsai et al. (2003) showed that 10,000 mg/L solutions of citric acid removed 99.999% of heterotrophic bacteria from surfaces in a simulated potable water system. Citric acid has also been used as part of a passivation system for stainless steels (Meltzer and Jornitz, 2006).

Although formaldehyde and glutaraldehyde have been shown to be effective agents against biofilm bacteria in water systems, their use is now extremely limited due to occupational safety and environmental disposal concerns. More recently, orthophthalaldhyde (OPP) has been evaluated as a biofilm treatment agent (Simoes et al., 2003). OPP is reportedly less toxic than other aldehyde compounds, and has been used for sterilizing heat-labile medical devices. The results of studies with *Pseudomonas aeruginosa* biofilms showed that OPP was effective at inactivating cells, but did not effectively remove biofilms from the underlying surface.

As with the type of chemical treatment, the compatibility of water system components (including microporous membrane filters) should be verified with the

TABLE 3 Chemical Treatment Regimes

Treatment agent	Dosage regime	Treatment time (h)
Quaternary ammonium compounds	300–1000 mg/L	2–3
Chlorine	50–100 mg/L	2–3
Peracetic acid/peroxide	0.02–0.05% (v/v)	2
Iodine	50–100 mg/L	1–2
Hydrogen peroxide	10% (v/v)	2–3

Source: From Mittelman (1995).

appropriate manufacturers. Handling and disposal of water treatment and other chemical agents require a familiarity with local health, safety, and environmental regulations.

The so-called bacterial regrowth phenomenon following chlorine treatments of purified and potable water systems is likely a function of biofilm formation rather than resistance in the classical sense of antibiotic resistance (Wolfe et al., 1988; LeChevallier et al., 1990). A combination of the polyanionic nature of many EPS moieties coupled with the inherent biocide demand associated with extracellular slime matrices may act as an inhibitor of various treatment agents. To date, the most effective chemical agents used in the control of bacterial biofilms in pharmaceutical grade water systems have been the oxidizing compounds such as chlorine (Rutala and Weber, 1997), hydrogen peroxide (Kim et al., 2000; Kim et al., 2002), and peracetic acid (Gorke and Kittel, 2002). Table 3 lists some of the more commonly applied chemical treatments along with typical dosage regimes.

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Steam Sterilization of Filters

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INTRODUCTION

Filter membranes variously described as 0.2 or 0.22 μm by their manufacturers have been in routine use for about 30 years for the sterilization of pharmaceutical drug products. Indeed, they are fundamental to the success of many aseptic production processes where the final product cannot be rendered sterile by heat treatment (steam autoclave) or other means. Equally critical to the success of any aseptic process where the sterilization of a liquid pharmaceutical product is achieved by filtration is the ability to sterilize the filter assembly. For disposable filter cartridges contained within rigid stainless steel filter housings, this means sterilization using pressurized steam. In the early days of their use, such filters were sterilized in a steam autoclave, after which treatment they were connected to associated pieces of downstream equipment such as filling machine components, using aseptic "sleight-of-hand" manipulations. As part of initiatives to improve the safety and reliability of aseptic manufacturing processes, many of these manipulations have been eliminated by the use of in-line steam sterilization procedures, in which the assembled processing system is supplied with pressurized steam under appropriate conditions to render the interior sterile.

More recent trends in the Bio-pharmaceutical industry have been to increase the use of fully disposable equipment and systems, especially for biologically sourced materials whose precise composition may not be entirely consistent. Using disposable materials eliminates the need to clean a lot of processing equipment and hence avoids both costly validation of cleaning and many of the risks associated with cross-contamination and product carry-over between batches. Sterilizing filters are therefore available as disposable plastic capsules comprising the filter element integrated in an outer casing. For safety reasons these cannot generally be sterilized by in-line steaming and must be sterilized by autoclaving, however in the context of fully disposable systems this does not present a handicap to their use. The improvements in aseptic handling technologies represented by laminar air flow environments and flexible film barrier isolators are heralding the return of (improved) aseptic manipulations to the sterile manufacturing process arena.

Steam sterilization cycles impose very great stresses on disposable filters, which are generally constructed using plastic materials whose melting temperatures are not far removed from those employed for their sterilization. Sterilizing grade filter assemblies can and should be tested for their integrity. Suitable procedures are validated by filter manufacturers to enable reliable non-destructive testing of sterilizing membranes

incorporated into disposable cartridge and capsule designs. These methods are sensitive to membrane defects and deviations from specification; they will also identify filters that have been damaged by handling procedures such as steam sterilization, a procedure that accounts for up to 80% of all genuine failures of filter integrity (as opposed to false test failures occurring through some incorrect application of the test procedure). For disposable filter cartridges installed in steel housings, the integrity of the whole filter assembly also depends on the condition of the housing seal faces, gaskets and connections.

Effective and reproducible operation of steam sterilization cycles requires an understanding of important aspects related to the system design and construction as well as the key principles of a sound operating procedure. These factors are essential if the sterilization cycles are to be validated successfully (Millipore Corp.). Thermal sterilization of equipment, including filters, can be validated using all the methods and analyses currently employed for autoclave filters (Lewis 2002), so that a Sterility Assurance Level can be assigned to the sterilization process. Sterilizing filtration systems regularly attract the attention and suspicion of regulatory authorities, in part because the same procedures cannot be applied to the process of sterilization performed by the filter for the drug product itself.

This chapter discusses practical design and procedural information about in-line steam sterilization, including some aspects of cycle design and validation. Operating principles important to the success of a sterilization cycle are described so as to provide a practical systematic approach suitable for readers to use in method development for individual applications. The operation of more complex systems can be based on the same principles, so that procedures can be derived for double in-line sterilizing filter systems, simultaneous sterilization of ancillary service filters or downstream equipment such as a receiving vessel or filling machine. Reference is made to autoclave sterilization only in so far as it applies to the sterilization of filter cartridges and assemblies. It is not within the scope of this chapter to deal in detail with specific procedures that have already received extensive coverage elsewhere (Agalloco 2000; Lewis 2002), such as the kinetics or validation of heat sterilization and filter integrity testing methods (Millipore Corp.). Similarly the guiding principles of operating an autoclave sterilizer are copiously elaborated elsewhere (Lewis 2002) and are not included here.

MONITORING STEAM STERILIZATION OF FILTERS

This section will review important considerations for monitoring the key parameters in the sterilization cycle for a filter assembly: the temperature, the pressure and the physical integrity of the filter membrane and assembly; this latter parameter cannot currently be examined until after the sterilization cycle has been completed (Millipore Corp.; Sartorius Technical Articles).

A primary requisite for controlled and reproducible steam sterilization is a clear understanding of the prevailing conditions in the sterilizing environment. In the enclosed space of an autoclave chamber it is relatively straightforward to create and demonstrate uniform conditions of temperature and pressure, notwithstanding the importance of load distribution and defined load patterns. The large body of published work on this topic bears witness to the extent to which the sterilizing environment within an autoclave has been characterized. By contrast it is more difficult to ensure uniform conditions throughout a dispersed and extended system of pipe work and equipment items such as filter assemblies, break tanks, particularly in the proximity of valves, pipe junctions, vents and drains (Cappia 2004; Cole 2006; Domnick Hunter Technical Centre; Kovamy et al., 1983; Millipore Corp.; Voorspoels et al., 1996).

Temperature

Successful steam sterilization requires monitoring of both temperature and pressure in the system, as it does also in an autoclave sterilizer. Indeed, in-line steaming may be seen as a more modern and desirable development from autoclave sterilization. The principles of thermal treatment kinetics and death rate calculations can be applied to in-line systems when subject to adequate measurement and control of the sterilizing conditions. The majority of sterilizing filter equipment can withstand the sterilization cycles employed in bio-pharmaceutical industry, which typically range from 121˚C to 142˚C (250–288˚F) for periods up to 1 h, according to the actual filter and system configuration. The upper temperatures used are limited by the materials of construction. Polypropylene is widely used for filter cartridge support materials ("hardware"), because it combines properties of strength and workability, while also being generally regarded as safe; it melts at about 165˚C (329˚F). Polyesters and polysulfones are also employed, but they generally have higher melting temperatures but may be less desirable materials as a result of their chemical nature and extractable substances. Thus sterilizing temperatures above about 145˚C (293˚F) create a significant risk to filter integrity. Softening of the filter support components facilitates distortion or collapse of the filter cartridge when excessive differential pressures are created across a filter assembly. When filters are subjected to higher temperatures (up to 160˚C, 320˚F), the filtration medium can separate from its (welded) seal to the cartridge end caps. Such temperatures can occur as a result of equipment failures or during process excursions, if not through choice.

Temperature mapping of an in-line sterilization system can be accomplished using thermocouples, in a similar manner to mapping of an autoclave load pattern. The issue must be addressed of whether to use surface thermocouples, which do not invade the item being sterilized, or thermocouples inserted into the system, which may affect heat penetration and distribution at the monitoring location. Similar to autoclave sterilizers, in-line systems should be mapped to allow routine monitoring of chosen locations ("cold spots"). In the case of in-line systems, however, it is also feasible to leave non-invasive thermocouples in place and so monitor temperature distribution in the system during every sterilization cycle. This may be particularly valuable for automated systems where the data can be logged and subject to statistical process analysis.

The temperature of pressurized steam is directly related to its pressure and can be accurately predicted when the steam is "dry" or free from condensate (water in the liquid phase) and "saturated" or 100% water vapor. A non-condensable gas such as trapped air will reduce the temperature of the steam from that expected at the prevailing pressure in saturated steam (Agalloco 2000; Custafsson 1998). Air is about 12,000 times more resistant to heat transfer than copper (Shuttleworth 2000), so it is a very good insulator and must be eliminated. There is very little difference in the specific mass of air and steam at the sterilization temperature and hence little gravitational effect to displace the air. The most practical way to purge non-condensable gases is by controlled steam flow through the system, ensuring a continuously advancing front of steam to displace air in front of it (Cole 2006). An autoclave sterilizer commonly employs a vacuum stage at the beginning of a cycle, more usually a series of vacuum pulses where each vacuum state is replaced by steam injection. The effect is to rapidly dilute residual air trapped in the load and minimize the risk of cold zones within it. A pre-vacuum may also be applied to in-line systems prior to the introduction of steam, but this may be unreliable in an extended or tortuous system that includes potential obstructions to the flow of air, such as filter membranes. To do so safely requires that the system is designed to withstand full or partial vacuum: many vessels are designed with thin walls to withstand positive internal

pressure only, and will collapse under atmospheric pressure if the internal pressure is significantly reduced. The seals must also be suitable and, to be effective, all in-line valves within the boundaries of the contained process system must be open during the vacuum phase and closed before steam is introduced. Air detectors are employed on autoclaves, ranging from a simple tube with a thermocouple mounted high on the machine to more sophisticated capillary tube devices with optical sensors. Such installations on an in-line aseptic process system would constitute crevices and be regarded as a risk to sterility in the system.

Temperature is also reduced by accumulation of steam condensate (liquid water) in drains and low points. Water has a high heat capacity—a layer of static water 1 mm thick is equivalent as an insulator to a layer of copper 500–600 mm thick (Shuttleworth, 2000). Contact between steam and water will tend to condense the steam further and exacerbate the problem, hence the need to remove as much excess water as possible from the system.

In addition to being concerned about factors that lead to reduction of temperature and the risk of creating cold spots in the system, it is also important to ensure that the temperature is not excessively high so that filter cartridge materials are not inadvertently damaged. Steam introduced too rapidly from a high pressure supply main can create much higher temperatures than may be desired as a result of superheating (Agalloco 2000; Shuttleworth 2000) (see below, "Steam Quality").

Pressure

An automated system may use a mix of gauges and electronic pressure transducers (or transmitters) mounted at strategic locations identified during the validation mapping study. Pressure sensors should be of sanitary design, generally employing a diaphragm membrane to protect the process system from the interior of the device. As previously noted, fully automated systems may use sensors for temperature and pressure at a greater number of locations for recording of data throughout every sterilization cycle, so that the information can be included in on-going analysis of process performance.

Pressure in a filtration system is monitored using pressure gauges, ideally on both sides of each filter. This permits determination of differential pressure across each filter, allowing the user to monitor the progress of each towards blocking. It is important to protect filter assemblies from significantly elevated differential pressures because, in combination with the temperatures of steam sterilization, severe damage can be caused to disposable filter cartridges. Filter vendors recommend appropriate limits for maximum differential pressure across a filter under steam sterilization conditions; these are typically 300 mbar (approximately 4.5 psi) in the forward or normal direction of flow and 200 mbar (approximately 3.0 psi) in the reverse direction, for temperatures up to 142˚C (288˚F). Some vendors may also offer filter cartridges specially designed to tolerate greater differential pressure during steam sterilization, although the majority of process systems may not require them. It is the author's view that most systems can be effectively sterilized within a maximum differential pressure of 300 mbar (4.5 psi) across the sterilizing grade filter and that filters claimed to withstand greater values act principally to encourage the use of poorly controlled sterilization procedures.

A greater degree of control is possible during monitoring of steam sterilization if the pressure sensor is also capable of reporting a vacuum in the system. The volumes of steam and its resulting condensate water are in the ratio 846:1 [for steam at 122˚C (252˚F)]. The change from the gaseous to the liquid phase can take place very rapidly and such condensation of steam is termed "collapse." A vacuum is created if the steam is not replaced by a non-condensable gas such as air or nitrogen. The effect of such a vacuum

may be to draw in potentially contaminated air from outside the system, via an inadequate seal or from the drain lines; it may also create an excessive and unexpected differential pressure across the filter, which, when combined with the prevailing temperature and residual steam pressure on the other side of the filter, can cause the filter cartridge to be seriously damaged—either crushed ("collapsed") or expanded—leading to loss of membrane integrity.

Inadequate flow of air through the vent filter to replace the condensing steam, for example, if the filter assembly has not been correctly sized, may also lead to a more explicit and dramatic demonstration of the term "steam collapse." For without suitable protection by means of a protective rupture device (bursting disc) or by a failure to design the vessel to withstand a vacuum, the vessel itself may be caused to collapse by the excessive differential pressure, amounting to 2 bar (30 psi), between the internal vacuum and the external atmospheric pressure. Some vessels are only capable of withstanding a vacuum as little as 50 mbar below the exterior ambient pressure. The event itself may take only a few minutes but the effects are costly and time-consuming and will cause some degree of embarrassment.

Filter Integrity

In an aseptic process the filter is the principal means of sterilizing the product being manufactured or protecting the previously sterilized product and the process itself from contamination. Tests of filter integrity are essential to confirm that the filter cartridge or assembly is undamaged in order that it might perform to the specification claimed by its manufacturer and demanded by the user.

Test methods have been developed and qualified under laboratory conditions by the filter manufacturers (Cole 1995; Docksey et al., 1999). These tests generally require the micro-porous filter membrane to be pre-wet with a suitable test fluid (pharmaceutical process solutions can be used, subject to qualification by the filter supplier) and are based on principles of diffusion ("forward flow," "pressure decay") or liquid displacement ("bubble point"). The relative merits of different test methods have been exhaustively covered in the literature and by filter manufacturers, to which sources the reader is directed. The tests should be performed using methods recommended by the filter manufacturers, most of whom make automated test equipment available. There may be minor differences between manufacturers in terminology and specific filter test parameters or limits; there may even be minor variations in the methodology of the test itself, which where possible the manufacturers will include in the design and programming of their specific devices; however, it should not be assumed that the use of a particular type or brand of filters necessarily dictates the source or type of test machine used. As for all critical process monitoring equipment, an automated test instrument should be suitably validated. The manufacturer of the test instrument can reasonably be expected to provide detailed support for installation (IQ) and operational (OQ) qualification. Of greatest importance, however, is the performance qualification (PQ), which will determine the suitability of the instrument and the test procedure for the actual test conditions in the process environment. By these criteria, some test methods or instruments render themselves more appropriate than others to a given set of circumstances. Many test instruments currently available may be integrated into computerized process control systems, when consideration should also be given to cGAMP and related issues; for example, 21CFR 11 electronic signatures, where a manufacturer may erroneously claim compliance or such compliance is irrelevant to the application of the device.

Filter integrity tests may be carried out off-line, or they may be performed in situ for previously sterilized filter assemblies without compromising the sterility of either the filter or the aseptic process system. The test methodologies are generally applied from the upstream ("non-sterile") side of the filter and depend also on the integrity or freedom from leaks of the upstream part of the filter system, as far back as and including an isolating valve. The tests can confirm integrity of the filter and correct installation in its housing; they may also distinguish pore size (grade) of the filter as well, to the extent that a coarser (or more open) pore size filter should fail the test, although a finer pore size filter may not be detected and excluded from use. Implications of this eventuality may include premature blockage or (partial) removal of components from the process solution. In systems using a double (serial) filter configuration, maintaining sterility between the filter assemblies places additional demands on the integrity testing procedure.

Tests of filter integrity are generally recommended before use of the filter, while most beneficial (and the stated preference of regulatory authorities (EMEA, 2003; FDA, 2004) is to perform this test after sterilization but before use of the filter, to confirm that it has not been damaged by the steam sterilization procedure. A test performed after installation and before sterilization can provide reassurance to the user that the filter is undamaged "out of the box," so that the time spent sterilizing the system is not wasted. This results in the filter being wet prior to sterilization, a condition that requires additional precautions to be taken during the subsequent steaming procedure. Wet membrane filters can be partially or completely dried prior to sterilization using compressed air (or nitrogen) flow, or drying may be effected at the start of the steaming procedure by controlled use of (mildly) superheated steam. These procedures should be validated to determine the treatment necessary to achieve a specified state of "dryness" for the filter (e.g., dry weight *vs* wet weight) before the sterilization cycle is undertaken.

A test of filter integrity performed after use of the filter is essential (EMEA, 2003; FDA, 2004) as part of the pharmaceutical manufacturer's assurance of finished product quality for batch release. The stated regulatory preference being, then, to test filters after sterilization (before use) and after process use, the majority of this chapter deals with steam sterilization of filters from the dry state, as would apply when they are tested for integrity after sterilization. The different circumstances and precautions applicable to steam sterilization of wet membrane filters are briefly discussed.

CYCLE VALIDATION ISSUES

Biological Kill

In spite of, or perhaps because of, the extensive characterization of thermal treatments to achieve sterilization, regulatory authorities still look for validation data based on microbial kill to support a heat sterilization procedure. This means that some part of the validation study for a filter system will include the use of *Geobacillus stearothermophilus* spores. This type of study allows any localized heating effects caused by material differences to be identified, such as might be due to seals, flexible hoses or lining materials (glass, PTFE) used in vessels and pipe work of some specialized systems. Filter cartridges are fabricated from materials that generally conduct heat poorly and the closely folded structure of a pleated filter membrane must be allowed to heat thoroughly during the initial phase of the sterilization. A study (Voorspoels et al., 1996) comparing the effect of different filter orientations on sterilizing efficiency in an autoclave showed that the blind end of the core of a filter cartridge (the "sterile side") was least likely to be

sterilized when the cartridge was orientated with its open end downwards, as recommended by filter suppliers to facilitate drainage of condensate from the filter core.

Temperature mapping of a filter cartridge during steam sterilization highlights the localized effects of material and environment on heat distribution. Using thermocouple probes inserted from the outside of a cartridge and placed between the pleats of a filter may give information about the temperature in this location; however this approach has some limitations. It can be argued that the insertion of a metallic object in this way assists heat penetration, either by conducting heat itself or by creating an artificial pathway for steam penetration. The thermocouple is also positioned on the upstream ("non-sterile") side of the filter membrane; while the information may be interesting, it does not report temperature conditions and hence permit calculation of an F_0 value inside the filter cartridge where we subsequently expect sterile liquid to emerge during filtration. The latter argument applies to insertion of a spore strip in this location, as indeed a spore strip may also facilitate steam penetration.

Conversely, inserting a thermocouple probe accurately into the least accessible part of a filter cartridge is difficult, given the typically small size of apertures in the filter support core and the relatively dense packing of the pleated membrane at the surface of the core. To achieve similar positioning for a small biological indicator (BI, or spore) strip is to be regarded as excessively challenging, especially when the BI must afterwards be recovered aseptically from the filter following a sterilization treatment, so that it can be cultured to demonstrate complete kill of the bacterial spores with which it had been impregnated.

Regulators of the pharmaceutical industry have expressed doubts in recent years about the importance of demonstrating the ability to sterilize a small piece of paper and the relevance to an ability to kill micro-organisms contaminating the surface of items of processing equipment. A suspension of these same bacterial spores can be used to inoculate the surface of different materials (stainless steel, glass vials, rubber stoppers, etc.) and techniques have been developed to enable recovery and detection of surviving spores. It is a matter of common experience that spores on the surface of some materials (e.g., rubber stoppers) are more difficult to kill than those on a spore strip, a phenomenon related to protective effects and the properties of heat capacity and thermal conductivity of the substrate.

The use of spore suspension instead of traditional biological indicator (spore) strips presents similar physical obstacles in terms of practical handling and microbiological aseptic techniques. Spore suspension can be deposited easily into the pleated membrane from the outside of the cartridge. Even from inside the filter's core it may be easier to pipette a couple of drops of suspension into a small aperture than to lodge a spore strip through it. Aseptic recovery of a spore strip after the sterilizing treatment suddenly seems an almost feasible proposition when compared with that of recovering spore suspension under these circumstances.

Necessarily, then, any validation approach using spores (either strips or suspension) will involve a degree of compromise. The challenge is to design a strategy for evaluating sterilization of filter materials using spore suspension, in which the problems of handling entire filter devices are avoided but the geometry of the filter device is maintained and taken into account in the sterilization procedure. Currently available industrial sterilizing grade filter cartridges almost universally comprise a pleated polymeric micro-porous membrane contained within the structure of an outer handling cage and inner support core that make access to the filtration medium difficult.

A suitable technique involves creating a "window" in the outer cage by cutting through the cage material to form an aperture that gives access to the pleated filter

medium beneath (Fig. 1). A small section of pleated filtration medium is then excised from the cartridge and the pleats opened to allow the membrane to be seeded directly with the required quantity of bacterial spore suspension. After drying, the piece of pleated medium can be closed and replaced in its original location in the filter device, aligning the pleats in their original positions. By leaving some parts of the cage uncut on one side of the aperture, a hinge is created to enable the original geometry of the filter device to be maintained and ensuring that the excised section of filter medium remains in place during the sterilization process. The filter cartridge is thus prepared and assembled into its steel housing in a suitable laboratory and then transferred to the processing environment where the sterilization process takes place. The technique is no different in principle to the use of biological indicators to validate sterilization of other parts of a process system.

Following exposure to the sterilization procedure, the filter device can be returned to the laboratory environment where it can be handled aseptically and the section of filtration medium removed using appropriate microbiological techniques, to enable its transfer to a culture medium for recovery and detection of any surviving micro-organisms. This method offers some advantages:

1. The spore suspension can be applied equally easily to both sides of the filtration medium, enabling treatment of the more important internal, downstream surface.
2. The possible survival of bacterial spores can be determined without the need to culture the entire filter device, reducing handling problems and minimizing false positive results.
3. Several apertures can be cut in a single filter cartridge, ensuring that a variety including even the least accessible parts of the filter device are treated, thus meeting compliance requirements for evaluation of the worst case.
4. The original geometry of the filter device is maintained, so that conditions during sterilization are representative of the actual conditions in a normal sterilization cycle.

A similar approach can be applied to filter capsules, but collaboration with the filter supplier may be advantageous; the latter can provide samples of filter capsules in which the outer casing has not been welded shut, giving access to the filter element within.

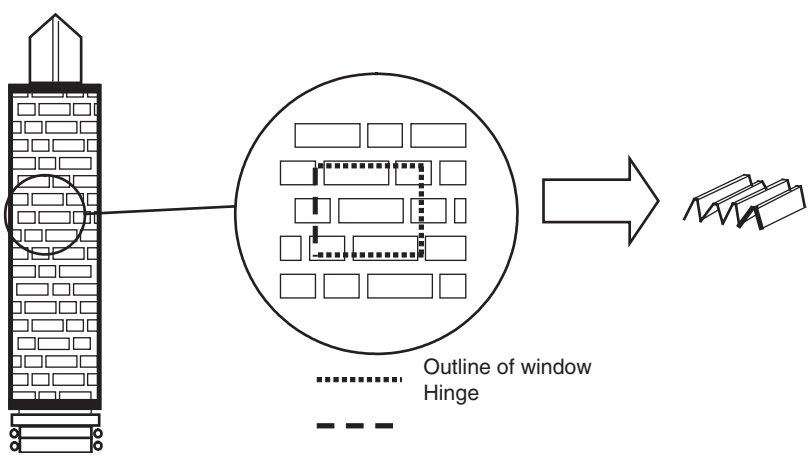


FIGURE 1 Excising membrane for treatment with spore suspension.

Steam Tolerance

Steam sterilization cycles will vary in terms of the conditions of temperature and pressure applied as a result of factors such as:

- size and configuration of the system and equipment being simultaneously sterilized;
- results of temperature mapping studies;
- quality policies requiring standardized conditions across a plant or organization.

Currently available sterilizing grade membrane filters often carry an impressive manufacturer's claim for steam life or tolerance. For liquid sterilizing (hydrophilic) filters, claims of withstanding up to 50 sterilization cycles or more of 1 h at 125°C (257°F) and up to 20 cycles or more of 1 h at 140°C (284°F) are not uncommon; however, with both the regulators and these same filter manufacturers urging single use of critical sterilizing grade filter cartridges, such claims should be interpreted appropriately by users of the filters. For gas sterilizing (hydrophobic) filters, which may be used repeatedly with intermittent re-sterilization (e.g., between each manufacturing batch), claims may reach in excess of 150 h cumulative steam exposure at temperatures up to 140°C (284°F). In this case, it could be presumed that such a claim might justify 300 sterilization cycles of 30 min each, but this is not the case. The major stress experienced by the filter cartridge is not during the actual sterilization period but the changing temperature conditions that occur at the beginning and end of the sterilization cycle. Expansion and contraction of the filter's polymer components places stress on the joints or welds, such as between the filtration membrane and the cartridge end caps or in the side seal that joins the two ends of the pleated membrane. Thus, the actual number of steam sterilization cycles undertaken represents a more meaningful interpretation of the validation.

For both hydrophilic and hydrophobic filter types, manufacturers make available validation documentation ("validation guides") to report the results of their laboratory-based qualification studies and justify their steam tolerance claims, which may be considered as a form of OQ carried out by the supplier. They nonetheless provide a guide to the relative robustness of filter cartridges and, taken in this light, the claims and supporting documentation are an aid to filter selection by the user (4). The data should be regarded with some caution, as even the most apparently robust and carefully selected filter could be damaged at the first application of steam in a poorly controlled in situ sterilization cycle. It is therefore incumbent on the filter user to perform an in-process evaluation (or PQ), in which the filters are exposed to realistic steam sterilization conditions and appropriate in-process limits are applied.

Steam tolerance claims are also provided by manufacturers for capsule filters. These disposable assemblies may be autoclave sterilized but are not generally suitable to be in-line steam sterilized. Some capsule products are claimed to be capable of in-line steaming, however the combined effects of rigorous health and safety procedures and the negative impact of litigation following an industrial accident have ensured that stainless steel filter assemblies remain the industry-standard for steam-in-place processes.

The tolerance to steam sterilization conditions of polymeric materials used for filter construction is based on the properties of the specific polymer and the included anti-oxidant additives. It is also important to consider the conditions experienced by the filter in the sterilization environment, before, during and after the validated sterilizing period. During the period of heating, when steam is first introduced, a mixture of steam and air (at elevated temperature) presents enhanced conditions for

oxidation (embrittlement) of plastic components. During in-line steaming this period is relatively short, while equipment-sterilizing autoclave cycles generally employ a pre-cycle pulsed vacuum stage to eliminate air from the load. The validated sterilizing conditions assume the complete exclusion of air from the process line or autoclave chamber, posing no oxidative risk to the filter materials. Necessarily, sterilization using validated mixtures of steam and air are excluded from this assumption. Furthermore, such sterilization cycles are not recommended for use with polymeric sterilizing grade filter cartridges and capsules. Following the sterilization period, cooling of the filter is likely to be accelerated for reasons of process convenience and time-saving. For a filter steamed in-line, the steam can be replaced by compressed air (of which more, later in this chapter), although this creates a hot air environment during the period of cooling. Nitrogen may be used instead to provide a non-oxidizing environment; this gas is certainly to be preferred for hydrophobic gas filters that, as noted above, may be validated to withstand a significant number of repeated steam exposures during a validated operating lifetime. At the end of the sterilizing period in an autoclave (equipment or porous-load cycle), it is common to pull a vacuum to remove all the steam and flash-evaporate residual moisture. The autoclave chamber may then be flooded with compressed air to restore atmospheric pressure; this creates an oxidizing environment that may last a considerable time, as a result of the mass of steel used to fabricate the chamber and its inherent heat capacity. Thus, manufacturer claims for steam tolerance of filter cartridges and capsules under autoclave conditions may differ from the claims made for the same filter cartridges when sterilized using in-line steam.

For all practical purposes, the risk to filter integrity posed by oxidative conditions is negligible in single-use applications, but it is increased when filters are subject to repeated sterilization cycles. A disposable filter capsule or an in-line filter cartridge might be claimed to withstand 25 autoclave or in situ steam cycles, respectively. While this may be the case, to validate such a number of sterilization cycles would invite the question why so many re-sterilizations came to be considered necessary. Rather than seeking to implement such a limit for a membrane filter employed in a single use application, a pharmaceutical manufacturer would undoubtedly be better engaged in establishing process and stock management procedures to ensure that exposure to such a number of steam cycles is neither required nor experienced.

CONSIDERATIONS FOR AUTOCLAVING

Although the objectives are similar to those for in-line steaming, putting a filtration device into an autoclave chamber is different in a number of respects. In common with the autoclave sterilization of any other item, there is the question of how to prepare the filter, wrapping it to protect the sterilized device from contamination when it is removed from the autoclave following the sterilization cycle. The wrapping must be porous to allow steam and air penetration but prevent ingress of bacterial contamination. This in itself is not a problem and has been well studied, however a sterilizing grade filter is susceptible to damage by excessive differential pressure generated across the membrane at elevated temperature. Thus, the inlet and outlet of the filter assembly must permit the free movement of air and steam and, equally important, neither must be susceptible to occlusion as a result of changing conditions during the autoclave cycle. Two particular changes can occur that are of special significance: generation of condensate (water) and softening of polymer components.

Condensate

Steam porous wrappings are liable to become damp during an autoclave sterilization cycle, although this may be controlled by appropriate techniques for applying the wrappings. The orientation of the filter assembly should be such that condensate generated within it will drain freely, to prevent its accumulation and subsequent formation of cold spots (Voorspoels et al., 1996). Since all apertures of the filter are commonly wrapped (inlet, outlet and vent or drain valves), irrespective of the filter orientation at least one such wrapping is likely to become saturated with water.

Also susceptible to saturation by condensed water is the hydrophilic membrane of a liquid-sterilizing filter element. The membrane of a filter that has been tested for integrity prior to sterilization by autoclaving may already be wet and thus resist the passage of steam and air. Under such circumstances the free passage of air and steam through apertures to both the upstream (inlet) and downstream (outlet) sides of the filter assembly is essential. If the wrapping that protects one or other side of the filter membrane becomes saturated with water at the same time as wetting of the filter membrane, those stages of the autoclave cycle involving pressurization (steam or air) or evacuation of the chamber may create a differential pressure between the two sides of the filter membrane and/or the autoclave chamber.

For example, suppose that during the sterilizing period at the required temperature and pressure, the filter assembly shifts position allowing condensate to accumulate in the inner core ("outlet" side) of the filter cartridge. This water can saturate not only the filter membrane but also the wrapping on the filter outlet. Where a length of flexible hose has been attached to the filter outlet (the distal end is covered with protective wrapping), the water could drain into this tubing. At the end of the sterilizing phase, during the reduction of chamber pressure by cooling, rapid exhaust or, worse, a post-cycle vacuum, the pressure in the inner core of the filter will tend to diminish more slowly and create an over-pressure. In this case the pressure difference is more likely to rupture the protective wrapping on the filter (or distal hose) outlet, either by expulsion of steam from inside the filter or by pressurized expulsion of condensate water accumulated in the outlet hose. While the (typically stronger) filter membrane may not be damaged in these circumstances, the sterilization cycle has failed because subsequent sterility and aseptic handling of the autoclaved filter assembly cannot be assured.

The orientation of the filter assembly becomes more important when considering the sterilization of a hydrophobic membrane filter. In the case of the hydrophilic membrane, condensate accumulating in the core of the filter can drain, whatever the orientation of the assembly, by permeation through (and wetting of) the porous structure of the membrane. A hydrophobic membrane specifically excludes the passage of water—a key property in the use of such membranes for gas filtration, contributing to maintenance of high gas flow rates through its resistance to wetting under adverse service conditions. In the above example, a differential pressure exerted across a hydrophobic filter membrane in the opposite (reverse flow) direction may cause condensate water to be forced into the membrane or its adjoining layers of support/protective materials. This would not necessarily damage the filter but can present difficulties for either integrity testing (after sterilization) or immediate placement in service.

Softening

The high temperatures experienced in a steam sterilization cycle result in softening of plastic filter components. Commonly used polypropylene materials melt at temperatures

from 165°C (329°F) and exhibit considerable softening at typical sterilizing temperatures. A disposable filter element, while usually robust at normal room temperatures, can be easily crushed using a (gloved) hand when heated to 121°C (250°F) or above. Disposable filter assemblies (capsules) can suffer damage as a result of deformation of the plastic materials during autoclaving.

1. An object resting against or placed on top of a filter capsule may be sufficiently heavy to deform the capsule during the cycle, thus making the capsule no longer safe to use under pressure or perhaps changing the capsule's shape so that it no longer fits correctly in its normal operating position. Attention to procedures for autoclave loading will prevent this problem.
2. Plastic filter capsules are commonly attached to stainless steel vessels by means of sanitary tri-clamp connections. One side of the connection (from the vessel) will usually be stainless steel while the other connection (the filter capsule) is plastic (typically polypropylene). The joint is secured with a stainless steel tri-clamp and an elastomeric gasket. During steam sterilization, differential expansion of the two flange materials and the gasket leads to distortion of the plastic flange on the filter connection, so that it no longer seals correctly. The effect is often exacerbated by a tendency to overtighten the clamps and is also exaggerated by the great disparity in heat capacity of the two flange materials; the steel components remain hot for a long time after the plastic filter materials have cooled. Use of the strong plastic tri-clamps currently available will eliminate most of the steel mass in this situation; alternative plastic connections to the vessel should be considered, for example, PTFE or polypropylene.

It is important to consider the sizing and duty of small disposable hydrophobic filter assemblies used as vents on equipment assemblies during both autoclave sterilization and normal operation. The sizing affects not only the likelihood of damage being caused to the filter but also the risk of damaging or destroying the equipment assembly itself. The following examples illustrate important considerations:

3. Small portable steel receiving vessels for sterile product are sterilized by autoclaving; a vacuum-cycle is employed before and after the sterilization period, to aid steam penetration and post-cycle drying. A small hydrophobic vent filter capsule (with a membrane area of approximately 500 cm^2) is used to enable venting during filling of the vessel and pressurization to empty it. The filter capsule is secured to the top plate of the vessel by means of a short length of flexible silicone hose that joins hose-tail connectors on the top plate and the filter outlet. The inlet to the filter capsule does not need to be wrapped, because the filter membrane protects the interior of the system from airborne contamination after sterilization. When the equipment is ready to be sterilized, the entire assembly is placed into a large autoclave chamber; the silicone tubing is sufficiently strong to support the weight of the dry filter capsule in an upright position. A test of filter integrity performed after use of the equipment revealed that the filter membrane was damaged and examination showed the filter had experienced an excessive differential pressure at elevated temperature in the reverse flow direction.
   At the temperature of the autoclave sterilization cycle the silicone tubing had softened. At the same time some steam had condensed in the filter capsule in the early stages of the cycle. The combined effects of increased filter weight and softening of the tubing caused the filter to collapse from its upright position; the end result was occlusion of the tubing where it became kinked between the filter

and the vessel (Fig. 2A). Thus at the end of the autoclave cycle, during rapid exhaust of steam pressure from the chamber by the vacuum pump, pressure could not initially be vented from the vessel, so while the chamber pressure may have approached a vacuum (0 bar abs) the inside of the vessel remained under steam pressure of 1 bar gauge (2 bar abs). The effect of the chamber vacuum would be to flash evaporate condensate, including that inside the filter capsule, resulting in loss in weight of the filter and cooling (hardening) of the silicone tubing. At some point, the filter would then rise and the tubing return to a vertical position, opening the kink (occlusion) and causing a rapid escape of the 2 bar differential pressure of steam contained within the vessel and damaging the filter membrane. When the autoclave chamber was opened, all appeared normal. An appropriate solution to this problem was to mount the vent filter capsule on a rigid outlet tube, at an angle that ensured free-draining of the filter core irrespective of the orientation of the vessel (vertical or horizontal) during autoclave sterilization.

4. A filter equipment assembly is prepared as follows (Fig. 2B): three hydrophilic (liquid-sterilizing grade) filter cartridges of 10 inch length (total membrane surface area approximately 2 m^2) are installed in a single filter housing. The outlet of the

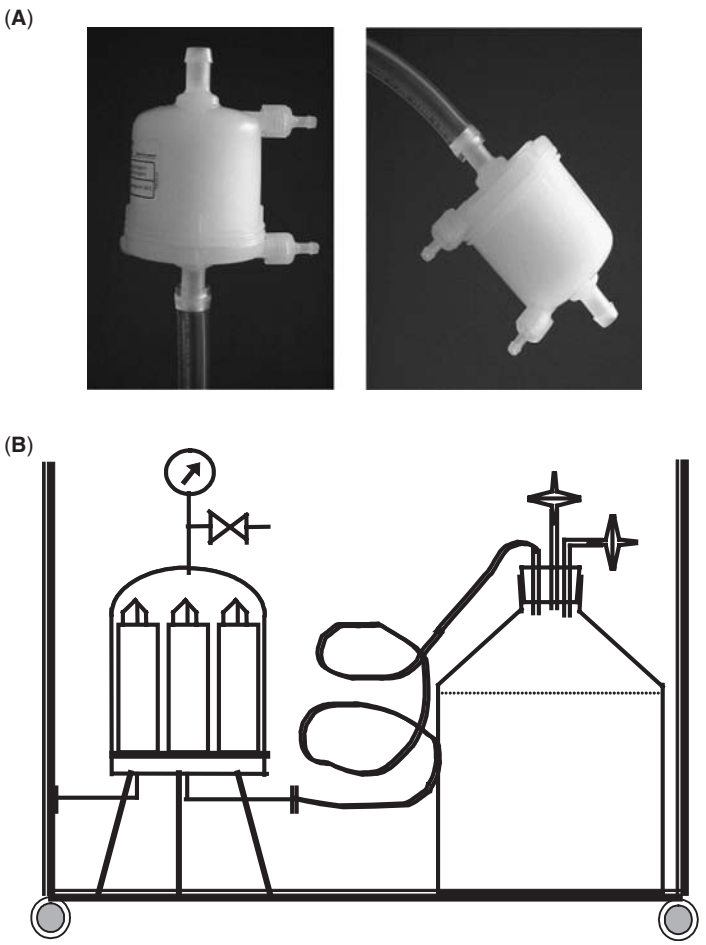


FIGURE 2 (**A**) Softening and occlusion of upright flexible tubing during autoclaving. (**B**) Inadequate sizing of vent filters for autoclaving of large equipment items.

filter housing is connected by 2 m of narrow bore (13 mm id) silicone hose to a hose tail connection atop a 25 liter capacity glass receiving bottle with a clamped rubber stopper and containing a small volume (200 mL) of purified water. Also connected via the stopper are two small vent filter assemblies with hydrophobic membranes (diameter 50 mm each, total surface area about 39 cm^2). One vent filter is mounted with the disc orientated horizontally and the other with the disc vertically. The autoclave cycle involved use of vacuum both before and after the sterilization period. On some occasions the glass bottle was destroyed during the cycle, either by exploding or imploding, the latter being less likely.

Instances of broken bottles correlated with performing a test of integrity for the large filter assembly prior to sterilization. Thus at the start of the sterilization cycle the three 10 inch hydrophilic filter cartridges were wetted with water and impermeable to both air and steam at the pressures incurred in the autoclave. At this point of the cycle however, the two small hydrophobic vent filters were dry and capable of allowing evacuation of air from the bottle, albeit slowly. Subsequent passage of steam through these vent filters was supplemented by generation of steam in the bottle from the purified water contained in it. During the sterilization period, small amounts of water would have condensed in the two vent filters; combined with a sudden escape of steam from the bottle at the end of the cycle, these two filters blocked and remained occluded while a vacuum formed in the autoclave chamber. The resulting 2 bar differential pressure between the inside of the bottle and the autoclave chamber caused the bottle to explode. However, when the three 10 inch filter cartridges had not been integrity tested before sterilization, their membranes remained dry and provided supplementary venting capacity to the entire assembly. The appropriate solution to this problem is to select and mount a vent filter on the bottle with adequate capacity to manage worst-case conditions.

In both these examples, a detailed "what if ... " hazard analysis of the sequence of events during the autoclaving cycle, when combined with assessment of all potential risks to components of the filters or equipment assemblies, would have identified such systematic cycle failures before they occurred.

DESIGN OF IN-LINE FILTER SYSTEMS FOR STEAM STERILIZATION

Effective performance of in-line steam sterilization for a filter system depends on the suitability of design and construction of both the system and its support services. The importance of eliminating condensate water has already been highlighted; still better to minimize the volume of condensate water produced. For drains to be effective they must be efficient and appropriately situated relative to other components of the system. Correct positioning of drains (or traps), however, is insufficient by itself to ensure that the system remains free of excess water. Significant contributions to achieving an environment of dry, saturated steam also come from well-designed pipe layouts and careful attention to the quality of the steam supply.

Filter Assemblies

For in-line steam sterilization, filter assemblies are generally made of stainless steel. Some disposable filter capsules made of particularly strong plastics have been proposed as suitable for in-line steam sterilization, but the potentially serious implications of

in-process failure of such assemblies have favored use of steel housings containing disposable filter cartridges. This has not prevented the increasing use of disposable filter capsules at point-of-fill or in completely disposable filtration and filling systems, for which they are well-suited, presenting many advantages for process design, scale up, validation, compliance and operation (Cole 2007).

Thoughtful engineering, improved manufacturing methods and operating experience have greatly improved the design of steel filter housings during recent years. Internal surface finishes have improved to match those previously enjoyed by the external (visible) surfaces, while enhanced sanitary design of drain and vent valves has made cleaning easier and reduced the risks of batch contamination. Design of housing bases and plenum chambers of single and multi-cartridge housings has improved the draining of condensate and recovery of residual product. The trend towards manufacture of smaller batches of higher value products has encouraged the use of small disposable filter capsules as noted above, but the design improvements have also benefited the manufacture of very small steel filter housings, combining the advantages of in-line sterilization and lower repeat cartridge costs with use of small filters.

Filter cartridge fitments have standardized on designs with a double O-ring seal, to minimize the possibility of filter by-pass. Cartridges should be inserted with a turning motion, the O-rings being lubricated with a suitable fluid (commonly water) prior to installation. During steam sterilization any fluid trapped between the O-rings will be heated; a fluid more volatile than water, such as an organic solvent having a higher vapor pressure than water, will create a zone of higher pressure in the isolated space between the O-rings. To relieve this pressure one of the O-rings may be forcibly expelled from its location in the cartridge adaptor fitting; once displaced it may be damaged and in any case is unlikely to resume its correct position, risking the security of the double O-ring seal. Equally, water may be trapped between the O-rings; this is not a problem during sterilization and may even be desirable to ensure the presence of wet heat in this location, but it may be a problem after the sterilization period. As steam pressure around the filter is reduced and cooling begins, the base of the filter housing (in which the filter cartridge adaptor and O-rings are enclosed) remains hot. Steam in the space between the O-rings remains in vapor phase and at the corresponding pressure (e.g., 1 barg), while pressure outside this space is lower. Thus the same problem may arise: expulsion of an O-ring from its correct location. This can be prevented by replacing steam with a non-condensable gas at the end of the period of sterilization, a subject covered later in this chapter.

Condensate

When water changes from the liquid to the gaseous phase (steam), the phase transition (vaporization) requires input of heat energy: the latent heat of vaporization (LHV). When the steam contacts a cooler material, heat energy is transferred in a process of equilibration. As the steam cools and gives up its latent heat, it once more returns, or condenses, to the liquid phase (water). It is therefore unavoidable that water, referred to as condensate, will be generated in a process system during steam sterilization.

The volume of condensate produced depends upon a number of factors, related to achieving the required temperature and then maintaining it for the duration of the sterilizing cycle.

1. The mass of material or system to be heated.
2. The specific heat capacity of the material or system.

3. The starting temperature of the material or system and hence the temperature differential to be overcome.
4. The external temperature and hence the temperature differential to be maintained.
5. The rate of heat conductance through the material or system.
6. The rate of emission (radiation) of heat from external surfaces.

The majority of aseptic processing systems are made from 316L stainless steel or other specialized steel alloys. The thickness and hence mass of steel is frequently considerable to meet appropriate safety requirements or standard engineering design codes. These materials have relatively high heat capacities and therefore necessitate considerable energy input to achieve the required temperatures. The increase in temperature necessary to achieve sterilizing conditions is also important and operators of autoclaves know that the first cycle of the day, when the autoclave chamber is cold, always takes longer to reach preset conditions compared to subsequent cycles. When sterilizing a system by injection of steam into the line, however, it is usual to begin with the system equilibrated at the prevailing ambient temperature. Specific system components may be equipped with supplementary heating, for example, hydrophobic vent filters on large vessels may be heated by an external steam jacket or electrical trace heating. Such means of external heating may also be employed during processing to maintain a process fluid at elevated temperature, and the equipment can be used to facilitate steam sterilization of the system.

Maintaining the required temperature of steam during the sterilizing cycle requires a lower energy input than that required first to achieve this temperature. Plastic or elastomeric materials, such as used for reinforced flexible hoses, are poor conductors of heat when compared to stainless steels. Indeed, the ability to use external, surface-mounted thermocouples to monitor sterilizing temperatures depends in part on the relatively good thermal conductivity of the steel. Once the material has conducted heat to the external surface, emission of heat (or radiation) from the surface will take energy out of the system, energy that must be replaced in order to maintain a steady internal temperature for sterilization. The emissivity of the material may be controlled by an appropriate choice of surface finish and by the use of thermal insulation; the latter also improves operator safety for manual systems, but should not be intrusive or detrimental to aseptic management of the process. As will be seen below, it is important to ensure a region of exposed pipe close to the point of entry of the steam into the system, to prevent excessive temperatures caused by superheating.

The initial input and subsequent replacement of heat energy necessitate a flow of steam through the system. Steam flow through a filter is needed to admit steam to the downstream parts of the assembly or equipment (see more on this later) but is not required to achieve sterilization per se. Compare the operation of an autoclave, in which steam flows into the sterilizing chamber to create a pressurized environment but does not flow through the items being sterilized. Unless the steam itself is being filtered, therefore, the steam flow capacity of a filter assembly is important only when it provides the main route to admit steam to downstream equipment. In this case the risk to the filter incurred during each sterilization cycle would be better managed by provision of a second (downstream) steam supply rather than simply using a larger filter.

Condensate Drains and Steam Traps

Good design to make process equipment self-draining is therefore important and is commonly achieved using drains isolated from the process system by sanitary valves.

Drains may be open, with an air-break between the process drain and a tun dish or common drain line, or closed, where the process drain is connected directly to a common drain line with no air break. The former configuration exposes the operating environment to emissions of steam and hot water, as well as to product, during the filtration process; the latter is a contained system but liquid from the drain may be drawn back into the process system in the event of vacuum formation after sterilization (see the following section). Drains are convenient for small, manually operated systems, purging steam, air and condensate continuously and therefore entailing some energy loss. There may also be safety concerns associated with jets of steam issuing from open valves and operator training is vital to ensure that there is a clear understanding of how and when to operate the valves, and the required degree to which each valve is opened or closed. In a large system, perhaps spanning two or more floors of a building, careful coordination is needed between several operators to achieve the level of control required. Systems may be fully or partially automated using valves that are motorized or progressively actuated, with preset stops to control the extent of opening or in combination with orifice plates in the outlets to restrict the flow of steam.

Steam traps do not require operator intervention. They are available in different types suited to different applications within the system, such as draining of condensate from the steam supply or from the process line and filter assemblies. A commonly used type, suitable for these applications, is the thermostatic trap. It relies on some radiation of heat from its outer surface to open and permit the discharge of (cooler) air or water, but it closes under the passage of saturated steam. Thermostatic traps are not generally considered to be sanitary in design and they require a sanitary valve to isolate them from the process line during normal operation. Steam traps do not open and close progressively but rapidly change orientation from closed to open position. This has implications for the protection of filter assemblies from elevated differential pressures, and these must be taken into account during design of a steam sterilization system and procedure. Too large a trap will allow too great a flow of air or condensate and hence too rapid a change in pressure. As will be discussed later, rapid changes in pressure should be avoided as they put at risk the integrity of a sterilizing grade filter membrane. Nonetheless, a combination of actuated valve and appropriately sized steam trap is well suited to automation, as the trap behaves like an orifice plate to restrict flow.

Valves

Different types of sanitary valves exist for different applications. Diaphragm designs are generally preferred in Pharmaceutical and Bio-Processing applications while butterfly designs are commonly used in food processing systems. Butterfly valves allow essentially full-bore flow in a pipe when fully opened but they are difficult to operate when fine control is required; diaphragm valves permit fine control but cannot give full-bore flow.

Valves should generally be opened and closed slowly (and *carefully*) during a steam sterilization cycle to avoid rapid changes, as noted above. An exception to this principle occurs when an excessive differential pressure occurs across the filter membrane and it is necessary to correct the situation quickly. In a manually operated system the appropriate use of valves can be managed through training of operators in the procedure and its underlying principles. When designing and building an automated a system, it has been this author's experience that greater importance is often given by consulting engineers to the cost of actuated valves rather than ensuring suitable controls to protect the function of the most critical component of the system—the sterilizing grade

filter assembly. Progressively actuated valves are often preferred for fine control, but alternative approaches are also successful. For example, a normally closed pneumatically actuated diaphragm valve opens when compressed air is admitted by a control valve, enabling the diaphragm to be lifted against a spring that holds the valve shut. If the air control valve is pulsed during valve opening, compressed air is admitted in small doses and the diaphragm valve will open slowly. The diaphragm valve is closed when this compressed air is released via a vent valve, and similar pulsing of this vent valve allows progressive closure of the diaphragm.

As important as the rate of valve operation is the sequence of valve operation. This is straightforward for an automated system, since the controlling computer will be indifferent to the sequence and relative locations of each valve. For a manually operated system however, training of personnel must ensure a clear understanding of the principles underlying the standard operating procedure, so that the correct speed and sequence of valve operation is a matter of intuition rather than blindly following a written method. Similar training should be considered for those involved in programming or designing the operating sequence for an automated control system.

Valve locations and operating sequence must also take into account that it is not enough to clean or steam as far as the back of a closed valve; it is necessary always to pass through the valve, to ensure that all contamination is removed from the gasket and sealing faces and that these places are then sterilized.

Steam Quality

The configuration of the steam supply and quality of the input steam are important to the success of a sterilization cycle and the condition of the process equipment. Steam made from high purity water in clean steam generators is highly corrosive to stainless steel. In some cases substances are added to the water feed to reduce the corrosive effects of the steam. Dissolved gas in boiler feed water (normally air) will be driven off when the water boils, leading to the presence of non-condensable gas in the steam. This air can accumulate in the steam supply and creates a risk of forming insulating-air pockets in the process system during steam sterilization.

So-called dry saturated steam has the full quotient of latent heat for the corresponding saturation pressure and has a dryness fraction (17) of 1, that is, it comprises 100% dry saturated steam and 0% water; while a dryness fraction of 0.95 is equivalent to a mixture of 95% steam and 5% water.

Superheated steam carries more energy than predicted by the saturation vapor pressure; the extra energy is carried as heat. It is usually generated by a drop in pressure, such as through a reducing valve, orifice plate or other restriction to flow, when the pressure of the steam is reduced but its energy content remains the same. A lower pressure of saturated steam results in a lower expected temperature, however if there has been no opportunity for this surplus energy to be lost from the closed system by radiation, the energy remains in the steam and a higher temperature results. This happens if a steam supply pipe feeding the process line is very efficiently insulated or if the process line is located very close to a high pressure steam supply main. The effect of temperatures unexpectedly reaching 170˚C (338˚F, equivalent to 7 barg steam pressure) or more on polypropylene filter components that melt at 165˚C (329˚F) are both fast and catastrophic. The extra energy in superheated steam, on first entering a process line, will vaporize any available condensate water, while the remaining excess energy will increase the temperature of the system components. It is therefore perhaps not such a bad

thing if the steam that initially enters a process line is usually carrying some condensate and that heating of the cooler line results in the formation of further, sometimes-abundant quantities of condensate.

Steam supply pressure should be regulated locally (but not adjacent) to the equipment being sterilized or more remotely using a lockable pressure regulating device. The presence of a dedicated regulator is important for several reasons:

1. To ensure that a general purpose service regulator is not inadvertently adjusted during the sterilization cycle, perhaps invalidating the procedure.
2. The regulator provides a significant reduction in steam pressure compared to the supply main, for example, from 7 barg down to 2 barg, so that the pressure in the filter system during sterilization is not affected if steam is drawn off from other points on the supply main.
3. The regulator may also provide an opportunity for the high pressure steam to expand and cool, to avoid superheated steam. There should be adequate opportunity for expansion and radiation of heat energy between the regulator and inlet valve for the equipment being sterilized; interposition of an expansion volume not only serves this purpose but also acts as a buffer to protect the process line from sudden rapid changes in steam supply pressure.

Pipe Work

In many plants the configuration of service pipe work (e.g., steam, compressed gas) bears little resemblance to either the original installation or the preferred state. Modifications, additions and deletions of points of use result in pipe-runs that are no longer required but that, for reasons of expediency, it was considered unnecessary to remove at the time. A length of pipe that ends in a closed valve or sealing device is sometimes called a *dead leg*. Dead legs can arise when a piece of equipment or a point of use is taken out of service; the steam pipe is sealed but not removed back as far as the active supply main and as a result steam condenses in the pipe. The resulting water may also accumulate behind the isolating valves at other points of use, creating cold spots, or the water may flood into equipment when steam is drawn from the supply. Similar problems may also occur if the active point of use for steam sterilizing a filter system is the last point of use at the end of the supply main. Filters are more vulnerable and more difficult to steam sterilize when wet and it is good practice to install a drain and steam trap immediately prior to the local pressure regulator or steam isolating valve. Some steam supply systems intentionally include a dead leg after the last point of use, fitted with a steam trap to ensure the active part of the supply main is always free of condensate.

An incorrectly designed fall in the pipe work or inclined filter housing can create low points adjacent to a filter assembly, or the weight of a filter assembly can depress an inadequately secured pipe and achieve the same end result. If water is allowed to collect at a low point, steam will condense more easily in the collected water; under the right conditions water may then be re-entrained in the flow of steam, spraying droplets into the filter assembly and wetting the filter membrane. Significant factors in the formation of such standing waves are the angle and diameter of the pipe, the volume of condensate and the flow rate of steam. It is therefore important to incline pipe work away from critical equipment items and towards carefully placed drain points.

GENERAL PRINCIPLES OF OPERATING CYCLES

Inadequately controlled steam sterilization procedures are the principal cause of damage leading to failure of integrity tests for sterilizing grade filters and, as noted above, differential pressure during steam sterilization is a major factor in such damage; its avoidance will significantly reduce the risks associated with the sterilization process. A small amount of differential pressure is essential to provide input of replacement heat energy to maintain sterilizing temperature. This is especially true at the beginning of sterilization when the tendency to form condensate is greatest, while the equipment components are being heated.

It is also important to maintain forward movement of steam through the system and thus displace residual air forwards to the next available drain or vent outlet. In systems having more than one steam supply the inlet pressures of each supply must be regulated so that there is no tendency for reverse flow of steam, which can easily damage sterilizing grade filters and which may also trap pockets of air between two advancing steam fronts. This issue becomes increasingly important for the steam sterilization of two sterilizing grade filters installed in series, or for filters with wet membranes where associated downstream equipment must also be sterilized in the same operation.

Process control during steam sterilization is achieved through applying the correct sequence of valve operation. Any standard operating procedure dealing with steam sterilization must include instructions for operating valves on both upstream and downstream sides of the filter. Deviation from the specified sequence may affect the efficiency of the procedure, for example, by not steaming adequately through valves, by driving excess condensate into the process line and by causing reverse steam flow or a differential pressure across the filter. The importance of training is again emphasized, to ensure that operators understand not only what actions are required but also the reasons why they are required. In an automated system the risk of a process deviation is reduced, subject to correct programming of the control sequence.

PRINCIPLES OF IN-LINE STEAM STERILIZATION IN A SINGLE-STAGE FILTER SYSTEM

This section follows the sequence of valve operation for a single-stage filter system, illustrated in Figure 3. The key operating principles underlying steam sterilization of this filter are explained and can be applied to more complex configurations. The steps described are designed to offer maximum protection to the sterilizing grade filter; this purpose should be borne in mind when modifying any parts of the procedure to meet the requirements of other components of a process system.

1. *All valves closed*. A safe starting position with all the valves closed is a good reference point to start all steaming procedures. It is safe for both operators and equipment. In an automated procedure, electronic feedback can be used to ensure that key valves are functioning correctly; if not, the cycle cannot continue.
2. *Prepare system downstream of filter: Open valve C*. Opening valve C enables steam to pass through it. The system on the downstream side of the filter remains closed at valves D & E, protecting the filter from a high differential pressure when steam is introduced by formation of a cushion of air (see step 5).
3. *Prepare system upstream of filter: Open drain H, drain J and vent G*. Opening all upstream drains and vents will allow air and condensate to be expelled when steam

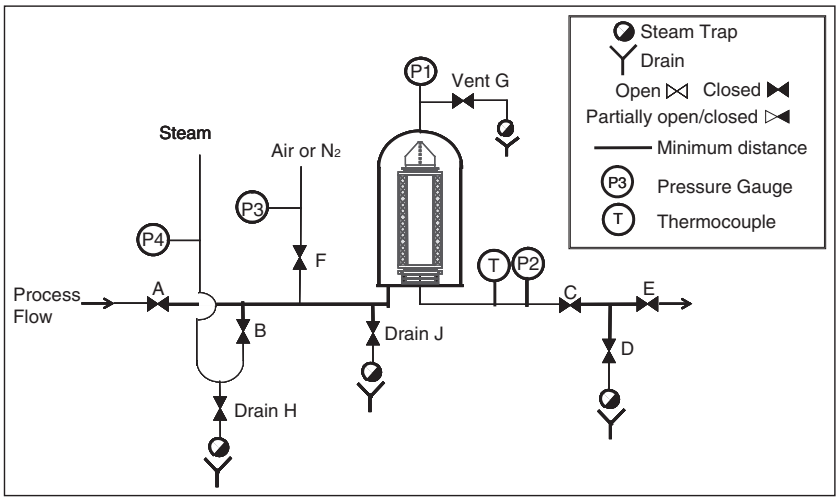


FIGURE 3 Single-stage filter system.

enters the system. Steam traps in the drains are non-sanitary and are therefore isolated by sanitary valves. A steam trap is considered essential at the position of drain valve H to prevent an unnecessary loss of steam and a reduction in the available pressure through an open drain valve. The steam supply pipe descends below the level of the process line and rises to the isolating valve B, a sensible precaution to ensure that condensate is purged *before* steam enters the process line. The connection at vent valve G to a steam drain or trap may be adaptable to allow connection of a filter integrity test machine or of a flexible hose used during priming of the filter system.

4. *Prepare the steam supply: Preset steam pressure P4 = P2 + 300 mbar; partially close valve H (if required).* The steam supply is prepared (or checked) by setting the local control regulator to the required pressure, typically about 300 mbar above the pressure required to give the specified sterilizing temperature in saturated steam at the thermocouple T mounted downstream. For the purpose of this example it is assumed that this is the coldest point in the system, determined by a temperature mapping exercise. Partially close drain valve H when steam issues freely from here; this is not necessary if a steam trap is fitted as it will close automatically.
5. *Admit steam: Slowly open valve B to admit steam; partially close valve J.* Valve B admits steam slowly and free of condensate because the supply line has been pre-heated and purged through valve H. Steam condensate formed as the process line is heated will first reach drain valve J, where it is expelled under pressure.

   Differential pressure created by flow of steam across the filter must be controlled, either by restricting the rate of pressure increase on the *upstream* side (valves B, J & G are open) or promoting a pressure increase on the *downstream* side (valves D & E are closed, creating a mixture of steam and compressed air as a cushion—see step 2).
6. *Purge air: Partially close vent G; (P1–P2) < 300 mbar; partially open drain D.* As steam chases air through the system, air is expelled and replaced by steam; partially close vent G. This will cause upstream pressure (P1) to increase; downstream pressure (P2) must also increase to minimize the differential pressure across the filter. To protect the filter from damage, a maximum differential pressure across the filter (P1–P2) of 300 mbar is typically recommended.

Now carefully open drain valve D on the downstream side, remembering that this will reduce downstream pressure and increase differential pressure. Drain valve D can be fully opened if a steam trap is fitted, as the trap will function as an orifice plate and restrict steam flow. If the differential pressure (P1–P2) increases towards 300 mbar, partially close valve D in order to control the rate of pressure change across the filter. Continue to monitor and adjust valve D until valve D is fully open and the steam trap is controlling pressure in the system.

7. *Sterilization period: Stabilize steam pressure (P1 = P2 = P4?); monitor temperature T and differential pressure (P1–P2) < 300 mbar*. Fully open steam inlet valve B and adjust steam pressure P4 using the local regulator valve if necessary, to achieve the required set temperature and pressure on the downstream side of the filter (monitors T and P2). In a small system with relatively little differential pressure, there is unlikely to be a significant drop in steam pressure through the system and P1, P2 and P4 may all report the same values.

   Sterilizing conditions have now been achieved and will generally be maintained without further adjustment of valves if the local pressure regulator on the steam is set at an appropriate value. Drain and vent valves (or steam traps) at H, J, G & D will continuously purge air and condensate. Temperature is monitored and recorded at T, which might also be located in the drain at valve D, depending on the needs of the actual system.
8. *End of sterilization: Preset air or nitrogen pressure, P3 = P4 + 200 mbar; close valves D, G, J*. At the end of the required sterilization period, compressed air (or nitrogen, N_2) should be used to replace the steam with a non-condensable gas. The use of carbon dioxide gas is not generally recommended as it is highly soluble in residual water lying in the system at the end of sterilization.

   Regulate the gas pressure to 200 mbar above the steam supply pressure. This overpressure prevents entry of steam into the air line; at the same time it provides a small but perceptible pressure increase in the system that does not risk causing an excessive differential pressure across the filter, should the membrane have become water-wet during the sterilization procedure. Each of the valves situated at the limits of the system are closed in sequence, starting on the downstream side—D, G, J, (H)—to ensure that the preset steam pressure is maintained in the system. Note valve B is still open for the same reason.
9. *Air ballast: Close steam valve B; immediately open air valve F*. Close steam supply valve B and immediately open gas valve F to admit the air or N_2. There should be an immediate increase in pressure at P1 and a similar increase is expected at P2 at the same time or within a few seconds. An increase in pressure at P2 confirms free passage of steam and air or N_2 through the filter membrane, indicating that the membrane is dry or at least not saturated with water.

   If P2 remains unchanged this indicates that the filter membrane does not allow passage of gas and is probably wet. Extra caution is required to prevent high differential pressures developing across the filter. If a membrane filter is always wet at the end of steam sterilization (or worse, at the beginning), additional measures are required to provide a source of sterile ballasting gas to the downstream side of the filter.
10. *Steam purge upstream: Flush steam from the system using air or N_2; open/close valves J and G*. Opening the filter drain J and filter vent G purges steam from the system and promotes cooling. Residual moisture is evaporated, removing water and also taking the LHV, as a form of forced cooling. Pressures at P1 and P2 should be monitored to ensure that they remain equal; the pressure will not be as high as

the original inlet pressure of the gas, and can fall as long as it remains above the atmospheric pressure of the surrounding environment. Air or N_2 passing through the filter to the downstream (sterile) side will be sterile if it has passed through a sterilizing grade filter membrane. If the filter is wet, P2 will be unchanged or start to decrease as the steam in the downstream system begins to condense.

11. *Steam purge downstream: Ensure P2 remains above zero (open valve E?).* If P2 approaches zero (atmospheric pressure), the filter is likely to be wet, the steam in the downstream side of the filter system has collapsed and there is now a risk of forming a vacuum if valves D & E are closed. If P2 falls below 0 barg, a vacuum has already formed and the risk is now of contamination ingress though a faulty seal, such as at drain D, hence the benefit of using pressure gauges or sensors that can display vacuum as well as positive pressure. When the filter is dry and allows free passage of air, the downstream valve E can be opened to purge steam from the system into the downstream line. A second source of steam or air can be introduced at the junction of valves C, D & E, to permit separate (and subsequent) steam sterilization of the downstream system and process equipment. Valve C will protect the filter from reverse flow and maintain the security of the previously sterilized filter assembly.
12. *Seal system: Close valves E, G & J; close valve F*. To seal the system, each of the valves situated at the limits of the system are closed in sequence, starting on the downstream side—E, G, J—and then the air or N_2 inlet valve F is closed. Using this sequence ensures that the pressure in the sealed system is equal to the preset air or N_2 pressure.

    The system may now rest under positive pressure to maintain sterility and prevent ingress of contamination if a seal were to be faulty, and to ensure that further cooling cannot result in formation of a vacuum as a result of the collapse of residual steam vapor.
13. *Vent pressure: open valve G; the system is ready to use*. To make the system safe and ready to use, open valve G to vent the excess pressure of air or N_2 from the upstream "non-sterile" side of the filter and then close all valves.

ASSOCIATED DOWNSTREAM EQUIPMENT

When downstream equipment associated with the process must be sterilized at the same time (Fig. 4 shows a receiving vessel with hydrophobic vent filter), we can consider a two stage approach. The first stage is the same as described above for a single filter system, until step 7 when the sterilization conditions have been stabilized and the set points for temperature and pressure are reached. For the second stage there is a choice. Valve M in Figure 4 is not required for sterilization but may be necessary during process operation, for example, to isolate the receiving vessel after filtration, or alternatively valve E may be relocated to the position of M to avoid two valves in this section of the line. This is an operational issue that does not greatly affect steaming.

Use Steam Supply No. 1 to the Filter

In this approach the single-stage filter system acts as the source of steam to sterilize the downstream equipment. Drain D removes condensate prior to the equipment (analogous to drains H & J for the filter). The same principles for preparation of the system should be

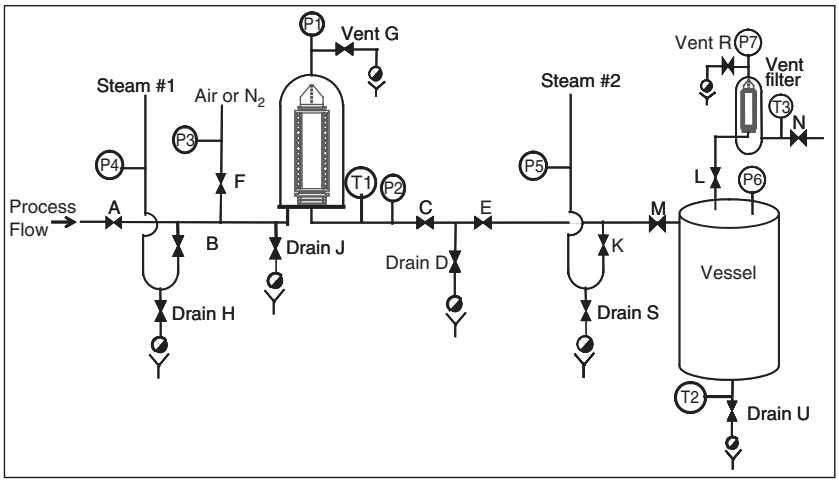


FIGURE 4 Single-stage filter system with associated downstream equipment.

applied as for a single filter system and when the downstream is prepared valve E is opened to admit steam (analogous to valve B for the filter). The sequence of operation, based on Figure 4, is outlined below; it is important to recognize that this sequence doesn't introduce any new principles but simply re-uses those applied to the filter. The feasibility of this approach depends on the relative sizes of the filter and the downstream vessel. If the former is too small or the latter is too big, the second approach is to be preferred (see below).

1. Open valve M and vessel drain U.
2. Slowly, fully open valve E to admit steam through valve M into the vessel. Condensate will drain from the vessel though drain U.
3. During this period of high steam flow through the filter, monitor and control differential pressure across the filter (P1–P2) by adjusting valve E. Monitor P6 for increasing pressure in the vessel and T1 to maintain sterilizing conditions at the filter housing.
4. When P6 = P2 the pressure in the vessel has reached the preset value for sterilization, partially open valve L to admit steam to the vent filter housing and open vent R.
5. Partially close vent R when steam issues from it (or employ a steam trap and isolating valve, as discussed above) and fully open valve L. This will now bring the filter assembly up to steam sterilizing pressure from the downstream side. The vent filter is therefore receiving a flow of steam in the reverse flow direction, for which a lower limit of acceptable maximum differential pressure may be recommended by the supplier. As was described above, however, the filter is protected from excessive differential pressure by a cushion of compressed air and steam in the normal inlet side of the assembly.
6. Carefully, partially open valve N to eliminate air from the inlet side of the vent filter assembly while maintaining the necessary back-pressure for sterilizing conditions in the vent filter.
7. At the end of the sterilization period, close each of the valves situated at the limits of the system in sequence, being valves N, R & U in the downstream equipment then valves D, G & J in the filter system.

8. Introduce air through valve F as before and repeat the same principles of air ballasting, expelling steam from vent and drain valves through the system by partially opening and closing in sequence valves: J, G, U, R & N.
9. At the end of ballasting close in sequence valves N, R, U, G, J, & finally F, to leave the system under pressure. Open valves G and N to vent the system for use.

Use (Supplementary) Steam Supply No. 2

This approach uses a second steam supply, regulated at a lower pressure compared to the first, so as to prevent reverse flow of steam back to the filter. The starting point for the procedure outlined below is taken as step 7 in the procedure for a single filter, when sterilizing conditions have been achieved in the filter assembly.

1. Partially open valve E to allow steam to pass into the downstream line but maintaining adequate backpressure so as not to compromise sterilizing conditions in the filter. Set steam pressure at P5 200 mbar below that at P2 (P5 = P2–200 mbar). Open drain valve S to purge condensate from the second steam supply.
2. Open valve K to admit steam to the line—the pressure is less than at P2 to prevent backflow. Fully open valve M and vessel drain U, admitting steam through valve M into the vessel.
3. When steam pressure in the vessel has reached that of the steam supply (P6 = P5), pressure in the vessel has reached the preset value for sterilization. Follow the same steps outlined above (4, 5 & 6), starting by partially opening valve L to admit steam to the vent filter housing and opening vent R.
4. Partially close vent R when steams issues (or install a steam trap and isolating valve, as discussed above) and fully open valve L. This will now bring the filter assembly up to steam sterilizing pressure from the downstream side. The vent filter is therefore receiving a flow of steam in the reverse flow direction, for which a lower limit of acceptable maximum differential pressure may be recommended by the supplier. As was described above, however, the filter is protected from excessive differential pressure by a cushion of compressed air and steam in the normal inlet side of the assembly.
5. Carefully, partially open valve N to eliminate air from the inlet side of the vent filter assembly while maintaining the necessary back-pressure for sterilizing conditions in the vent filter.
6. At the end of the sterilization period for the filter, which frequently requires a shorter time than used for the downstream equipment, close valve E (or C if valve E is not installed, as discussed above) and perform air ballasting as described for a single filter (steps 8–11).
7. At the end of the sterilization period for the equipment, close in sequence the valves at the limits of the system - valves N, R, U & K - then introduce air from valve E (or valve C) via the filter, which has already cooled and rests under compressed air pressure). Alternatively, compressed air can be introduced through valve N and the (now-sterile) sterilizing-grade vent filter, purging steam through vent R and drain U applying similar principles.

The size of downstream system for which a second steam source is preferred will depend on the system configuration, its heat capacity and the number of vent and drain outlets for steam, but may be in the range 5–50 times the size of the filter assembly. The relative size is also affected by the steam flow capability of the process line (hydrophilic)

filter. Larger downstream systems require a steam sterilization cycle that is much longer than is normally required for a filter assembly; using a second steam supply allows the filter system to be cooled after sterilization, ready to act as the source of sterile ballasting gas when sterilization of the downstream equipment is completed, should compressed gas not be available at the vent filter.

DOUBLE FILTRATION SYSTEMS

A double or two-stage filter system (Fig. 5) can be managed using a similar two stage approach to that applied to associated downstream equipment. Sterilizing conditions are established in the first filter stage; meanwhile the valve positions may be prepared in the second filter stage. The same two choices of approach are available and the same operating principles should be applied. Either use the first filter as the source of steam for the second or use a second independent steam source with the pressure regulated sympathetically with respect to the first. A second (sterile) gas source may need to be connected between the filters, for ballasting and to provide a means to perform a pre-use integrity test on the pre-sterilized system without compromising sterility in the zone between the filters.

Valve Requirements in Double Filter Systems

When there are two steam sources, the forward displacement of air by steam is essential to prevent entrapment of air pockets and reverse differential pressure across the filter. A Tee-configuration of valves was proposed between the filters, for example, in Figure 5 valves C, D & E. It is frequently considered undesirable to have a drain located in pipe work between two sterilizing filters (including, for that matter, a drain point incorporated in the design of the second filter housing) but experience suggests that it is effective to do so. Some more recent designs of sanitary diaphragm valves incorporate an integral drain located adjacent to the seal face (weir) making them convenient for condensate drainage in this configuration.

There are some benefits to this valve configuration, deriving from improved control of steam flow and enhanced condensate elimination. The configuration is essential if a second steam source is installed, allowing independent operation and sterilization of different sections of the system. Providing the ability to sterilize the

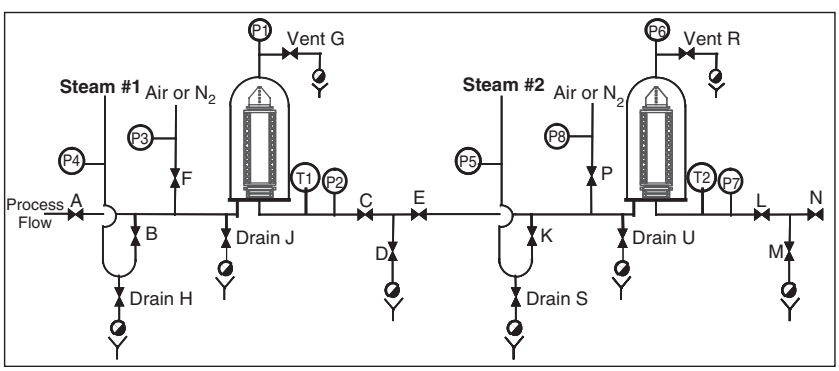


FIGURE 5 Two-stage (double) liquid filtration system.

filter separately from the remaining downstream equipment allows the use of sterilization cycle times appropriate to the filter, instead of subjecting everything in the system to the sterilizing conditions required by those components most difficult to sterilize. The simplified operation and improved control will also reduce the risk of the filter being damaged during sterilization, which in turn can simplify validation of the procedures used.

Figure 6 shows a hydrophobic filter labeled F2 that can be used to provide sterile air (or nitrogen) into the process line, but it can also be used as the second source of steam to sterilize the line downstream of process filter F1. Sterile gas can be introduced from valve F for ballasting the filter at the end of the sterilization cycle, while filter F2 continues to supply steam for sterilization of the system downstream of valve C. At the end of the sterilizing cycle for the downstream system, a large flow of non-condensable gas may be required to protect vessels against vacuum when the steam collapses; the liquid filter is often not sufficient for this purpose. The hydrophobic air filter (F2) is located downstream of the liquid filter and can be cooled and also used to feed sterile gas directly into the equipment. This enables satisfactory ballasting of the downstream system, but also allows a sterile gas purge to be used to recover valuable product from the line downstream of the liquid filter.

An alternative means to purge the line of product following filtration is to increase air pressure upstream of the process filter (F1) until the bubble point of the membrane is exceeded—the pressure at which liquid is displaced from the pores of the filter by overcoming the capillary forces holding the liquid in place. This pressure will be typically in the region of 3000–3500 mbarg, and carries risks, firstly that the filter membrane might be damaged by the procedure if it is carried out in an uncontrolled manner; loss of filter integrity at this moment, even if the filter was intact and functioning correctly during the filtration process, will result in loss or reprocessing of the batch. Secondly, the breakthrough of air across the wet membrane may cause carry over of microfine contaminants, previously retained by the filter membrane, into the recovered downstream product.

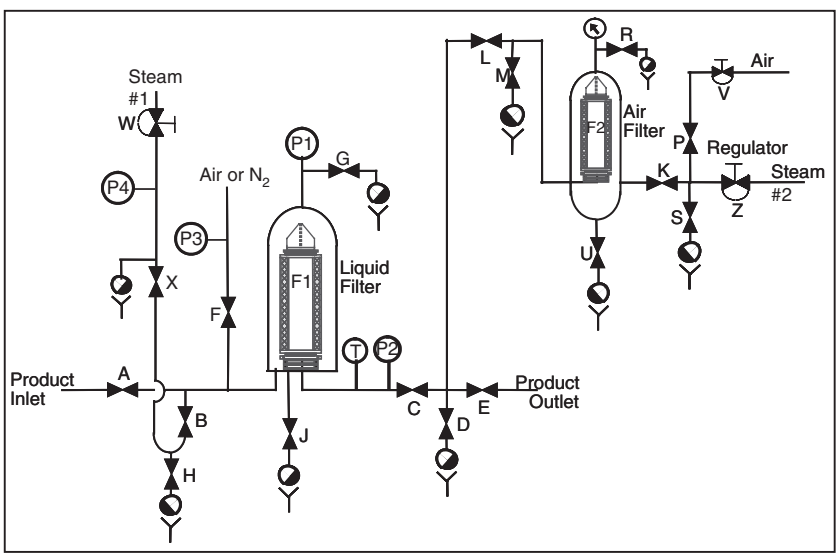


FIGURE 6 Another configuration of double filter system—suitable for wet filters.

Filling Installations with Double Filter Systems

Figure 7 is a schematic representation of a double filter system where a vessel has been fitted between the two filters to ensure a continuous feed of product under defined conditions to the second filter and aseptic filling equipment. This receiving vessel would necessarily be sterilized as part of the procedure to sterilize the filters and interconnecting system. Both liquid filters should pass integrity tests before and after use, in addition to the vent filter. Such a system is implemented on many types of filling machines using the Blow-Fill-Seal technology. The system shown in Figure 7, however, differs from the majority of installations in that a second source of steam is illustrated between filter A and the vessel. This second steam source minimizes the risks during steam sterilization to filter integrity and thence to product sterility assurance and process security, but it is not commonly employed. In some such systems, the first filter (A) and the vent filter (C) both provide steam to sterilize the vessel, filter B and the filling heads, an approach with merits.

Such a complex system requires monitoring in a number of key locations. Figure 7 illustrates possible locations for monitoring temperature and pressure, as well as provision of steam and gas supplies. The same principles as were adopted in the previous systems can be applied to the design of this steam sterilization procedure. Liquid filter A is sterilized using steam supply no. 1. When temperature at T1 has reached the set limit, steam no. 2 is admitted at lower pressure (P1–P5 = 200 mbar) to the vessel, draining condensate through valve S. Liquid filter B and vent filter C can be sterilized at the same time from steam supply no. 2, in combination with steam from supply no. 1 through filter A. Ballasting gas for filter A is introduced through valve F (P4), while for the vessel, vent filter C and liquid filter B, ballasting gas is supplied through valve N (P9).

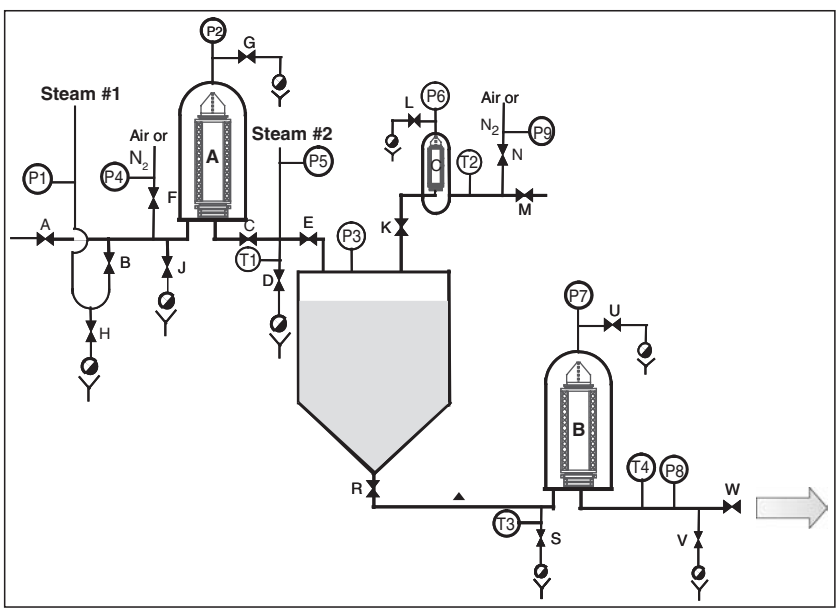


FIGURE 7 Double filtration in a filling system.

STEAMING WET FILTER MEMBRANES

Although pre-use testing of filter integrity is better performed after steam sterilization, there are occasions where this may be impractical, such as when the filter is used for a non-aqueous product where water must be excluded from the process. Equally, it is recommended that certain types of liquid sterilizing filter membrane materials should be pre-wet before steam sterilization, for example, to remove wetting agents or as an aid to preserving their hydrophilic characteristics. A sterilizing filter membrane must be fully wetted during current integrity test procedures and in this state will not allow passage of gases (air or steam) until the membrane bubble point pressure is reached. This requires a pressure typically in the region of 3500 mbarg, equivalent to 148˚C (298˚F) in steam and such conditions of pressure and temperature would be certain to cause severe filter damage.

Methods are available to limit the problems associated with a wet membrane during steam sterilization. The filter can be partially dried using air flow. After wetting (or integrity testing), compressed air is carefully applied until the bubble point pressure of the membrane is exceeded and then air flow is maintained until the membrane remains damp but allows free passage of gas. Alternatively, in the first phase of steam sterilization, controlled input of superheated steam may be used to partially dry the filter. In this case, the filter housing (with vent valve fully open) acts as an expansion chamber, allowing the pressurized steam to expand rapidly. Excess energy in the steam vaporizes water from the filter membrane and the steam vapor exits through the vent valve. Both methods must be validated to define the conditions necessary to achieve free gas flow, bearing in mind pre-wetting requirements of some membrane materials. Pre-weighed sample filters that have been water-wet are then dried by gas or steam flow, weighing the filter cartridge and performing measurements of pressure drop after different intervals of treatment as a means to determine residual moisture in the membrane.

Safe sterilization of a wet filter can be accomplished more easily when conditions at the filter surface are understood (Fig. 8).

1. Steam vapor arriving at the cool outer face of the filter membrane condenses, gives up the LHV and so heats the water held in the membrane pores, creating more water.
2. Pressure of steam builds on the upstream side of the membrane and this in turn raises the temperature in the environment around the filter cartridge. It must be done slowly however, to avoid creating excessive differential pressure across the filter membrane. As a result of steam condensation on the membrane, there is transfer of the LHV and the membrane temperature rises.
3. Conduction of heat through the water trapped in the filter increases the temperature of water at the downstream face of the membrane. When the water reaches 100˚C (212˚F) on the downstream face of the membrane, it begins to boil because the ambient pressure downstream in the filter core is atmospheric.
4. As water boils off the membrane it creates steam that takes with it the LHV, energy derived from the water in the membrane, so the membrane cools. In turn, the pressure starts to rise on the downstream side of the filter because the outlet valves are closed (the principle of the air cushion described above), so also raising the temperature.
5. When sufficient pressure has built up on the downstream side of the filter, a downstream drain valve can be opened slowly to purge the trapped air.

The conditions in the filter come rapidly to equilibrium as steam is generated, but this operation requires care as there is no free flow of steam through the membrane—rapid loss of steam through the downstream drain will result in sudden and excessive differential

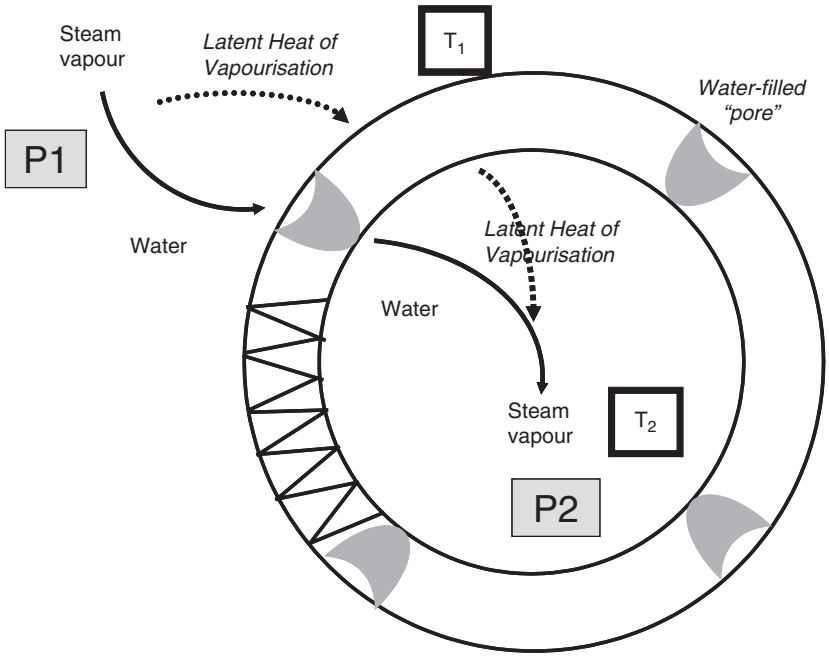


FIGURE 8 Steaming a wet filter membrane—*phase transition.*

pressure across the filter at the elevated temperatures of steam sterilization and the filter cartridge may be crushed.

Therefore, a wet filter membrane can be steam sterilized safely in-line. The operating principles and procedures are the same as previously described, with the important caveat that they must be implemented with a greater degree of care and control. Because neither steam nor air can flow through the filter at the prevailing pressure used for sterilization, care must be exercised when introducing steam and when air ballasting after the sterilization, especially on the downstream side of the filter. Raising the pressure of steam or air to exceed the bubble point pressure of the hot filter cartridge is not an option to be considered seriously. As discussed above, second (downstream) sources of steam and air should be used as a preferred means of achieving safe sterilizing conditions; preventing trapped air pockets by continuous forward displacement with steam is important but the problem of forming a downstream vacuum is avoided.

STEAMING FILTERS IN THE REVERSE FLOW DIRECTION

A number of filtration processes are allowed to develop and may thereafter be implemented to include the requirement to sterilize filter assemblies with steam in the reverse flow direction. This is not a practice to be recommended highly, especially by the filter suppliers, since filter cartridges are intrinsically stronger when subjected to differential pressures in their normal forward direction of flow (outside-to-inside). Nonetheless, it is a practice that is likely to continue, in some cases because there are good operational reasons why it is essential and in other cases because the additional cost and effort to design and build a system that avoids it was outside the scope of the project.

The principles laid out above should be applied. Additional care is needed to allow for the reduced strength of the filter in the reverse flow direction and greater attention must be given to testing integrity of the filters, both before and after use, to ensure that a batch of valuable product is neither committed to a damaged filter nor lost as a result of filter failure. Special attention is required for condensate elimination; filter assemblies don't generally have provision for drains on the outlet side and, in systems designed to maximize product recovery, condensate may drain away from the filter into the flow of advancing steam, being re-entrained in the steam flow and spraying the membrane with water. Ballasting gas should still be introduced from the normal inlet side of the filter, if possible, requiring a sterile gas filter but providing at the same time a source of gas to recover product from the outlet line.

TROUBLESHOOTING

The majority of filters that fail tests of integrity for reasons of genuine damage (as opposed to inadequate wetting, incorrectly applied procedures, poor installation or physical damage) incur damage during steam sterilization. Automated systems with data-logging equipment provide evidence of physical conditions in certain parts of the system. The data should form part of an investigation into filter damage so that reproducible causes of filter or sterilization cycle failure can be eliminated in future.

An invaluable aid to any investigation is the filter itself. It can be thought of as an eye-witness to the events leading to its own damaged condition and should be examined accordingly. All aspects of the filter's condition must be taken into account and filter suppliers may have considerable expertise in this area regarding their own products. The materials of construction have known physical properties and any observable changes in filter components can provide information about the equipment failures and process excursions that caused them. For example, while a high differential pressure at elevated temperature may crush (or collapse) a filter element, the appearance of the polypropylene components may indicate something about the actual temperatures experienced. At temperatures close to the melting point, some thinner polypropylene parts such as the outer cage will have the appearance of partial melting or flowing of the plastic; from a slightly lower temperature, a previously smooth and reflective surface may take on a non-reflective or frosted crystalline appearance.

A filter cartridge was used in the air vent line to a production autoclave chamber during one month of operation. It failed a post-use integrity test and its condition when the housing was opened is shown in Figure 9A. Initial examination suggested exposure to an excessively high temperature (about 160˚C, 320˚F), witnessed by the frosted and partially melted appearance of the outer cage. This was combined with high differential pressure applied in the forward direction of flow, evidenced by collapse of the filter cartridge. Indeed, the collapse was so severe that the walls of inner core remained about 3 mm apart and the normally flat blank cap at the top of the cartridge was concave. Another indicator of extreme collapse was that the very severe nature of the distortion of the cartridge end caps made it impossible to remove the filter from its seat in the housing. Subsequent review of the autoclave records revealed that, three days after the filter had been installed, an engineering team replaced a liquid-ring vacuum pump on the autoclave after it had functioned incorrectly; other critical components of the autoclave, such as the filter, were not checked at the same time. Meanwhile the validation group had reported problems with the chamber air detector, leading to significantly higher operating

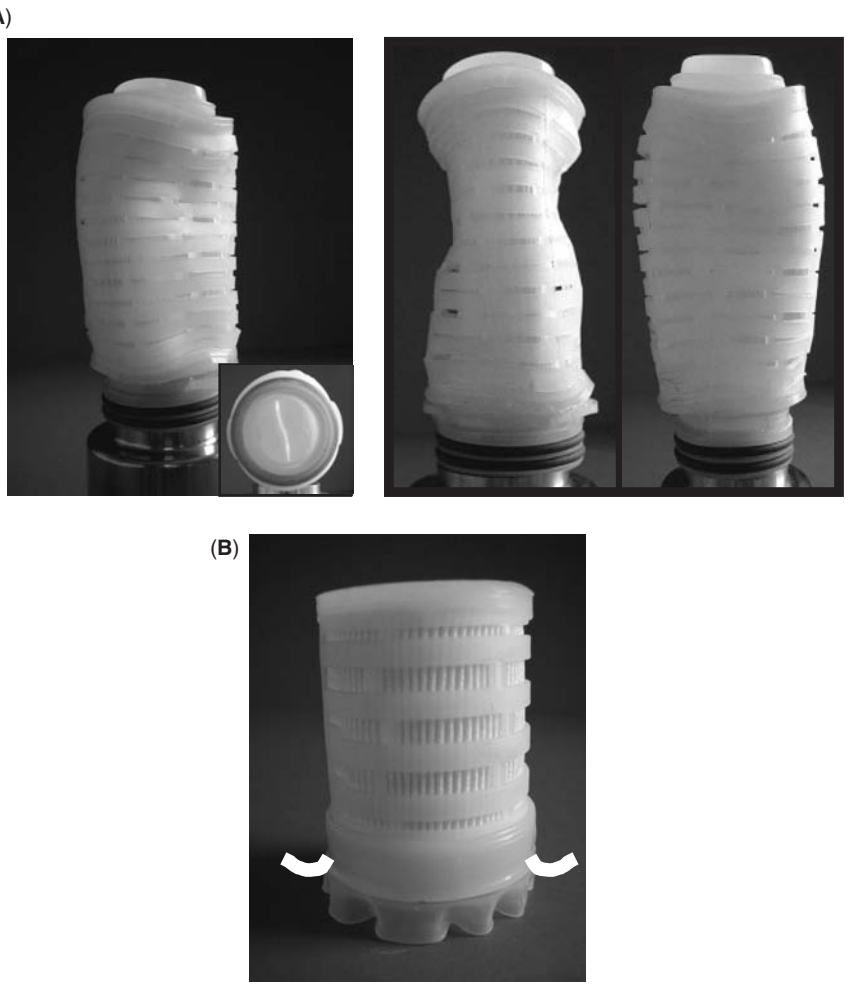


FIGURE 9 (**A**) Collapse and high-temperature exposure of a single filter cartridge. (**B**) Effect of reverse differential pressure at elevated temperature on a filter cartridge with a flange seal.

temperatures than required as the autoclave sought to achieve the set temperature in the air detector; once again, the filter was not examined. Similar thermal effects would have resulted from introduction of superheated steam, and at even higher temperatures above the melting point of polypropylene the filter may be reduced to a small dense ball of plastic lodged in the housing seat.

Depending on the filter cartridge configuration, the effects of high differential pressure in the reverse direction may be equally evident, resulting in a *ballooned* filter element expanding to assume the diameter of the filter housing. Figure 9B shows a small filter cartridge equipped with a flange seal design. During steam sterilization in the reverse flow direction, the filter became saturated with condensate as a result of inadequate provision for drainage. At elevated temperature the flange became soft until the applied pressure of steam built up behind the filter cartridge and propelled it to the opposite end of the filter housing; it is also possible to see the effect on the filter of its impact with the other end of the housing—the upper edges of the top end cap are beveled.

Operators reported hearing the sudden release of steam pressure in the housing and the sound of the filter hitting the end of the housing.

Damage caused by excessive differential pressure may be less obvious but equally detrimental to the performance of a sterilizing grade filter. The direction of perforation at the apex of a membrane pleat may be apparent from the orientation of the edges of the hole. Microscopic examination of the surface of the membrane can reveal differences in the depth of impressions left in the membrane by pressurized contact with the upstream or downstream non-woven support and drainage materials used in most sterilizing filter designs. High differential pressure applied from the upstream side of the filter will mean deeper impressions are visible on the downstream side of the membrane, and vice versa. (The membrane is the means of transmitting the pressure and it is forced into contact with the non-woven materials. The non-woven layers are too open or porous to be directly influenced by an applied pressure. When a filter element is crushed by excessive forward differential pressure [Fig. 9(A)], the outer cage appears to have been crushed in addition to the membrane and the filter core. This distortion of the cage however results from the shape taken up by the crushed membrane and core; the cage is forced to adopt a similarly wider, flatter conformation but is not itself crushed by the pressure.)

Multi-element filter cartridges are fabricated by welding together 2, 3, or 4 individual 25 cm long (ten-inch) elements; their collapse provides an appearance that is sometimes surprising (Fig. 10). The outcome of their exposure to a high differential pressure at elevated temperature is determined by the weakest of the individual elements. Although the real difference in strength between the elements may be negligible, the orientation of the plane of collapse of the first serves to determine the orientation of the remaining elements. Collapse of the first element results in planar distortion of its end

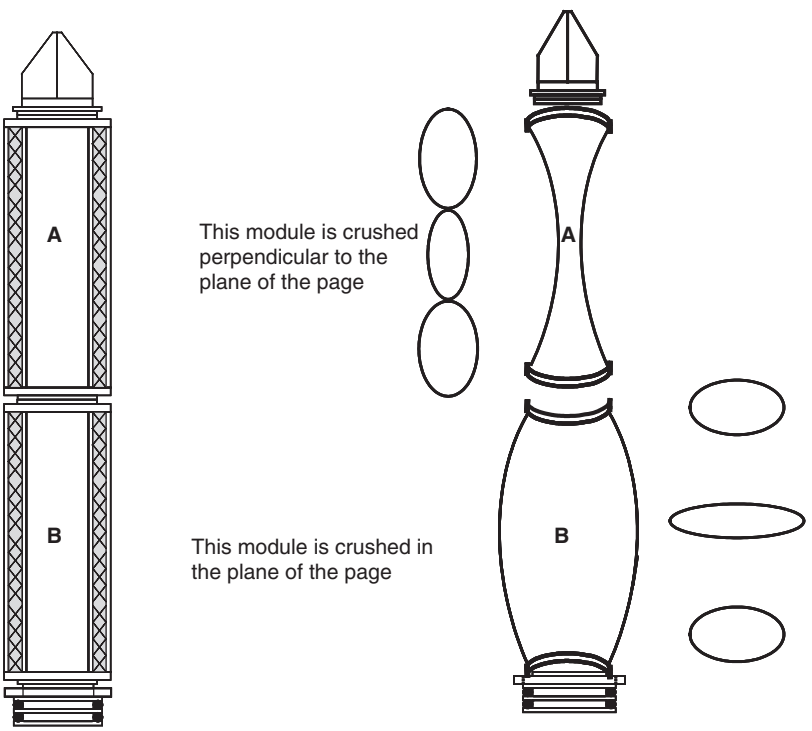


FIGURE 10 Collapse of a multi-element filter cartridge.

caps, the edges being drawn towards the centre of the element in the plane perpendicular to the plane of collapse. There is a corresponding outward distortion of the end cap in the plane of collapse of the element. The force exerted by this distortion of an end cap is transmitted to the end cap of the adjacent module, to which it is welded; the resulting distortion of the adjacent end cap is in the opposing sense, causing this element to collapse in a plane orientated perpendicular to the plane of collapse of the first element. The orientation of collapse of each element is therefore determined by propagation from its neighbor (Fig. 10).

Failure to provide adequate drainage of condensate can cause filter damage due to high differential pressure, either by saturation of hydrophilic membrane materials as described above, or by blinding of hydrophobic membranes. When the steam supply itself has insufficient condensate drainage, similar outcomes can be expected. When the steam supply arrives at its point of use (frequently) above a filling machine, it is important to ensure all condensate is purged from behind the steam isolating valve. It is also beneficial to ensure that condensate drains on and before filling machines (such as Blow-Fill-Seal systems) are adequately sized. When an un-drained steam supply is opened and several liters of condensate are driven under steam pressure from a 2 m length of 50 mm diameter pipe into the filling machine, the 13 mm diameter drain on a small hydrophobic gas filter housing (probably sufficient in most cases) will be overwhelmed. Build up of pressure is relieved by forcing water though the hydrophobic filter membrane, creating a hammering effect that will certainly damage the filter membrane and also creates significant vibration in the entire piping system of the machine.

Following steam sterilization, procedures have been discussed earlier to replace the steam with a non-condensable gas such as air or nitrogen. The consequences of steam collapse in the system can be severe.

1. Leaking seals, or those designed to prevent an escape of pressure from the system, may be caused to leak inwards in response to the vacuum formed when steam condenses, bringing external contamination into the sterilized system.
2. If a filter remained or became wet during steaming, compressed air introduced to the upstream side will not pass through the filter to compensate for the pressure fall as steam collapses on the downstream side. The result will be 1 barg air pressure (=2 bar abs) upstream and vacuum (0 bar abs) downstream, a net forward differential pressure of 2 bar across a hot filter. If the orientation of these events is reversed, the differential pressure will be exerted in the reverse flow direction.
3. Additional risks attach to systems with closed drain systems, that is, where the drain lines from the filters and pipes of the process system are sealed to the common outlet drains (welded or clamped joints). Formation of a vacuum in the process system will suck back liquid and contamination from the common drain. If another part of the process equipment (on the same or another floor of the building) is simultaneously undergoing solvent washing or alkaline cleaning-in-place, for example, these aggressive liquids may be drawn from the drain into the hot filter system, with catastrophic consequences for chemically incompatible filter cartridge materials.

CONCLUSION

Many factors of the design and operation of a process system can influence the steam sterilization procedure. A systematic approach to developing methods appropriate to the

system configuration is based on applying broadly similar principles to single filter assemblies, double filter systems and those with more complex processing equipment. Effective training for process operators must ensure both detailed knowledge of the individual steps of the operation and clear understanding of their underlying purpose and principles. When planning an automated system for sterilization-in-place, the same training and understanding may be required by those people involved in the design and programming stages of the project, or close involvement in the team of suitably qualified process and validation personnel.

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Ozone Applications in Biotech and Pharmaceuticals

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INTRODUCTION

Sanitization in any healthcare-related environment is one of the most important, if not the most important, aspect of patient care or product integrity. Virtually all of the advances in pharmaceuticals, devices, and treatment would be for naught if sanitary conditions could not be maintained and infections inevitably resulted because of, or in spite of, treatment.

Since the foundations of scientific microbiology were laid by Koch in the late 1800s, as a result of his Nobel Prize-winning work at the Institute for Infectious Diseases in Berlin, we have had a clearer understanding of the mechanisms of infection and disease. Koch's "germ theory" and his epidemiologic work were critically important in developing appropriate regimes for patient care and treatment. Over time, practical solutions for the maintenance of sanitary conditions developed which included appropriate application and use of materials and enhanced cleanliness standards utilizing heat as well as materials such as iodine, alcohol, formaldehyde, peroxide, ozone, and chlorine.

Chlorine has become the most common sanitant used for treatment of potable water in the United States and is also often used for non-potable water applications, both uses based on its oxidative strength and low relative cost. Within the pharmaceutical industry, it is common to see supplemental treatment using sodium hypochlorite (NaClO). More recently, for drinking water, chloramines have seen increased utility since chlorine-ammonia compounds are more stable, offering extended protection against microbial growth. Chlorine compounds however, can result in the development of trihalomethanes or THM's which are considered to be carcinogenic, and hence obviously undesirable. The development of THM's has been referred to as "induced contamination" (The Clean Technology) since the chlorine that is added for sanitization reacts with naturally occurring organic precursor compounds, such as humic and fulvic acids as well as algal byproducts, resulting in organohalogens, some of which are listed in Table 1. Many of these haloforms have the general formula CHX_3 where X represents chlorine, bromine, etc.

There are two possible explanations for the presence of bromide compounds, one being that bromine is introduced during chlorination as an impurity present in industrial grade chlorine. Alternatively, and more frequently, bromides present in raw water are oxidized to HOBr, which is highly reactive in the presence of organic matter

TABLE 1 List of Organohalogens

Chloroform	$CHCL_3$
Bromodichloromethane	$CHBrCl_2$
Dibromochloromethane	$CHBr_2Cl$
Bromoform	$CHBr_3$
Carbon Tetrachloride	CCl_4
Trichloroethylene	C_2HCl_3
Chlorobromomethane	CH_2BrCl
Tetrachloroethylene	C_2Cl_4
Dichloroethane	$CH_2Cl\text{-}CH_2Cl$

(The Clean Technology). Although ozone does not result in the formation of THM's, other disinfection byproducts of ozone, such as bromate, can be potentially hazardous.

The potential for trihalomethane formation (THMFP) "refers to the maximum quantity of THM's that will be produced by the precursors present in the water, taking into account the conditions most favorable to THM formation (excessive free chlorine combined with a 3 or even a 5-day contact time)" (The Clean Technology). Determining the potential for THM formation in raw water is of critical importance for the health and safety of the public as well as for the design and implementation of specialized treatment as is required for pharmaceutical water production. Additionally, ozone can be used in the form of pretreatment to oxidize THM precursors prior to the application of chlorine in a water treatment facility.

The use of chlorine as a disinfectant grew substantially after World War I, based primarily on cost, however chlorine compounds also offer the benefit of residual action as they continue their sanitizing action throughout distribution piping, up to the point of use in individual residences. This is especially important for large municipal distribution networks as regrowth could occur in the piping if localized disinfection was the sole method employed.

Heat, although impractical for residential use, except in emergencies, has been the most accepted and reliable method of sanitization utilized in healthcare applications, including surgical tool sterilization, as well as for biotech and pharmaceutical production. Dry heat, is effective for sterilization and also for depyrogenation using specialized ovens. Alternatively, wet heat can also be employed. Pure steam as utilized in autoclaves may also be direct injected into equipment and piping systems to provide effective sterilization. Heat can also be applied indirectly to raise the temperature of liquid product above 60°C, provided heat sensitivity is not of concern. The use of heat is extremely common as it can reliably eliminate most viable species of microbial contamination; however the cost of heat sanitization has prompted investigation and use of alternative methods.

Within the healthcare industry, water is the single largest commodity employed, finding use in product formulation, cleaning, and manufacture. Hence, control of bacteriologic purity is critical. As such, it is important to note that significant differences exist between requirements for potable water and for water associated with drug products. Potable water microbial standards, promulgated by the U.S. Environmental Protection Agency and defined within National Primary Drinking Water Regulations (NPDWR), allow for as high as 500 colony forming units per milliliter (cfu/ml), while the United States Pharmacopeia (USP) lists action limits for microbial contamination in varying grades of bulk pharmaceutical waters from 100 cfu/ml to 10 cfu/100 ml.

Microbial limits for products and excipients may be lower based on application, such that it is not uncommon for sterility to be required, especially for parenteral (injectable) products. Sterility can be accomplished using dry heat, wet heat, gas sterilization (i.e., ethylene and propylene oxide), ionizing radiation (i.e., gamma and cathode ray), and via filtration.

Although not recognized as a sterilizing agent, ozone has gained far greater acceptance in the United States for use in sanitization since it offers a number of advantages and is useful in a multitude of applications. It should be noted that acceptance in the United States has been slower and is less widespread than in Europe, as ozone use in Europe has been far more extensive with many long term applications.

Ozone, commonly designated O_3, is a gaseous tri-atomic allotrope of oxygen that is extremely unstable, and as a result it is one of the most powerful commercially applied oxidizing agents in use. Ozone can be formed in various ways with the most common commercial methods based on the application of electricity to oxygen. The relative oxidative strength of ozone can be readily seen in Table 2 making it more powerful than chlorine or peroxide.

As such, ozone is considered a hazardous material and must be handled in appropriate fashion. However, unlike heat that scalds on contact, ozone is somewhat more subtle in that its pungent odor and acridity is obvious even at levels well below Occupational Safety and Health Administration (OSHA) threshold limits for exposure. This is an obvious advantage as many deadly gases are odorless and hence difficult to detect.

Based on its reactivity, high concentrations of ozone can be explosive. Although concentrations at these levels are usually unachievable with standard commercial equipment, certain improper application of equipment and materials, such as carbon stripping of an ozonated gas stream (ozone in air); can result in an increased risk of fire or explosion.

Atmospheric ozone is created by ultraviolet irradiation however this method is generally too inefficient for most commercial purposes. For relatively large volume commercial production, various electric discharge technologies are the most commonly employed manufacturing techniques. The two most often implemented for pharmaceutical site production of ozone are termed “Corona Discharge” or “Cold Generation” whereby ozone is produced from a gaseous feed of either air or oxygen, and “electrolytic” where oxygen from the dissociation of pure water is the feed source. Each methodology will be discussed in additional detail in the following pages for the purpose of providing background information salient to the topic.

TABLE 2 Relative Strength of Ozone

Oxidizing agent	EOP (volt)	EOP vs. Cl_2
Fluorine	3.06	2.25
Hydroxyl radical	2.80	2.05
Oxygen (atomic)	2.42	1.78
OZONE	2.08	1.52
Hydrogen peroxide	1.78	1.30
Hypochlorite	1.49	1.10
Chlorine	1.36	1.00
Chlorine dioxide	1.27	0.93
Oxygen (molecular)	1.23	0.90

Abbreviation: EOP, Electrical oxidation potential.
Source: Stanley (n.d.) and Meltzer (1993, 1997).

Based upon its unstable nature and short half-life, ozone cannot be stored in containers for subsequent use, hence it is generated relatively close to the point of application or delivery, typically for immediate use. Ozone's half life has been estimated to be approximately 25 min at 20°C in distilled water (Meltzer, 1997) however many factors can influence its rate of decomposition, including temperature, pH, and the presence of oxidizable substances. As well, "ozone is only sparingly soluble in water, in general about 13 times more soluble than oxygen" (Meltzer, 1997), with a high rate of off-gassing anticipated.

As an oxidizing agent, ozone can be problematic relative to materials of construction, especially plastics, because of its basic ability to break double carbon bonds and as it degrades to hydroxyl radicals (OH° and HO_2°) to break higher carbon bonds, before finally reverting back to a simple oxygen (O_2) molecule. Ozone is also very aggressive in its attack of metallics, with the exception of gold, platinum, and iridium, as it creates metal oxides in the highest oxidation state. Alternatively, certain metal alloys can easily withstand ozone contact in typical concentrations including the class III, 300 series, austenitic stainless steels commonly specified by the biotech and pharmaceutical industries. As an unanticipated benefit, ozone's eventual degradation back to oxygen is thought to assist with maintenance of the passive film that forms on stainless steel, reducing the development of rouge and increasing the interval between repassivations. Further discussion of materials compatibility with ozone will follow in subsequent sections of this chapter and discussion of stainless steel passivation may be found in the chapter relating to stainless steel.

In addition to its oxidative capacity, ozone also serves as a disinfectant and a sanitant as it kills cells by lysing, or causing the cell wall to rupture. Ozone also has the capacity to decompose microbial byproducts, also known as endotoxin. Ozone is effective against all bacteria, virus, cysts, and spores in varying degrees depending on the concentration, contact time, and other physical conditions, serving as an excellent biocide.

Ozone is also rapidly degraded to oxygen by ultraviolet irradiation of the 254 nm wavelength, offering the added advantage of simple and inexpensive removal, using a technology already embraced in both the biotech and pharmaceutical arenas, and which itself offers an added degree of microbial reduction.

Ultraviolet irradiation, in addition to destroying ozone, has been shown to have excellent germicidal effect and based on wavelength and intensity is also suitable for reduction of; Total Organic Carbon or Total Oxidizable Carbon (TOC), chlorine, and organics. Dosing for ozone decomposition is usually in the range of three (3) times that used for bacterial destruction, with a 90 mJ/cm2 UV dose typically effective for removal of 1 mg/l ozone in high purity water to below detectable limits. The action of the UV radiation produces OH° radicals (short lived) and oxygen (O_2). It should be noted that ultraviolet bulbs cannot be manufactured such that only a single wavelength is emitted, hence bulbs with specific wavelength ratings may be predominant at those wavelengths, based on masking, however, other wavelengths cannot be completely excluded. Therefore, bulbs rated for ozone destruction also provide some amount of germicidal effect as well.

Ultraviolet equipment is also available with both low and medium pressure bulbs, albeit not from every vendor. The intensity of medium pressure bulbs allows for, in many instances, significant downsizing of the equipment and higher flows per bulb, however there is a trade-off in that the bulbs are significantly more expensive, control cabinets are substantially larger and require more power, and bulb life is shorter creating a situation where operating costs may offset anticipated savings.

When compared to heat, ozone offers a number of distinct advantages not the least of which is low relative operating cost. As a matter of fact, as the cost of energy continues to rise, current high energy practices such as heat sanitization (especially when subsequent cooling is required) will come under increased scrutiny, and ozone is likely to see more opportunities for use. As such, it is already of increasing interest to designers, manufacturers, and operators. This statement must be kept in perspective since it is not applicable to other industries especially commercial water treatment, where the comparison must be made to chlorine injection. In these cases ozonation may be considered more energy intensive and hence more costly.

There are no accepted standards for ozone concentrations expected to result in complete microbial elimination primarily due to the variance in water chemistry and microbial loading. Current practices vary from below 0.01 mg/l to higher than 2.0 mg/l (ppm) with the vast majority operating between 0.10 and 0.30 mg/l. The International Society for Pharmaceutical Engineering has issued a baseline guide for water and steam that indicates it is common for system ozone residual to be set between 0.02 and 0.2 mg/l (International Society for Pharmaceutical Engineering. 2001). Lower levels obviously result in lower cost operation and it has been noted that higher concentrations can often result in a shift of pH to the acidic side potentially compromising the ability to meet compendial standards. This is judged to be the result of reaction by the ozone with trace amounts of resident organics present but below the levels expected to cause failure based on TOC alone (Fig. 1).

The use of ozone for commercial pharmaceutical applications began in earnest in the United States during the mid to early 1980s, however there may have been earlier functional installations. After the initial barriers to its application were removed, the use of ozone grew slowly but steadily, gaining ground as a reliable sanitant, although it has yet to gain universal acceptance or quell all of its detractors.

Early installations at Lederle Labs (now Wyeth) and Richardson-Vicks helped to lead the industry by example and in spite of minor flaws, these and other systems were milestones for the use of ozone as well as the development of pharmaceutical water system technology. In its 1993 Guide to the Inspection of High Purity Water Systems, the FDA acknowledged the use of ozone, commented on its functionality, and through the use of examples noted limitations that can become problematic if not properly addressed, paving the way for ozone's further use.

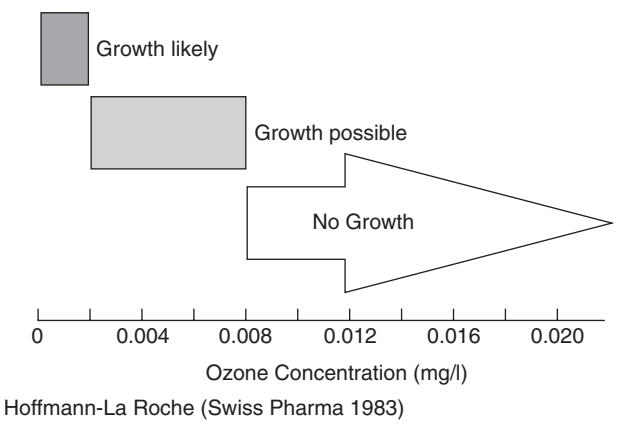


FIGURE 1 Microbial growth vs. ozone concentration (Hoffmann-LaRoche). *Source*: From Stanley (n.d.).

Ozone, unlike chlorine, leaves no residual after its decay to oxygen. Hence, any regrowth of organisms will be unimpeded, unless ozone contact is virtually continuous. The FDA Guide makes note of microbial contamination in ozonated systems and includes a system schematic in which ozone is apparently added only to treated water entering the storage vessel and also mentions another wherein for safety reasons, "ozone was removed from the water prior to placing it in their recirculating system" (U.S. FDA, 1993). These issues have been successfully addressed in current system designs with continuous ozonation of storage volumes and frequent ozonation of distribution piping, facilitated by enhanced controls that lock-out use-points during periodic sanitizations. Since distribution loop sanitizations can easily be automated and require little time, daily sanitizations are considered minimum with higher frequency preferred.

Unfortunately, ozone and its value have always been shrouded in controversy even to this day, apparently because ozone research has been limited and extremely focused. A search of the Internet yields literally thousands of articles relating to global warming ostensibly a result of depletion of ozone in the earth's upper atmosphere. There, ozone, produced by ultraviolet radiation, filters shorter wavelengths (< 320 nm) of ultraviolet light that would otherwise reach our planet's surface, potentially harming most forms of life. Depletion of the ozone in the stratosphere allows this radiation to pass and appears to also result in surface temperature increases with global repercussions. It has been postulated and is widely believed that the recorded ozone depletion is caused by "greenhouse" gases such as chlorofluorocarbons or CFC's as well as from automotive and industrial emissions rather than by normal cyclic planetary environmental activity. However, it should be recognized that there are also many who doubt that the changes in the ozone layer are the result of human activity, alternatively believing it to be normal climatic cycling. This debate, although interesting and of significant concern, is beyond the scope of this volume.

Interestingly, continued searches of the Web reveal a vast array of material that might be best characterized as lively debate covering a wide range of applications and issues relating to ozone beyond that of global warming. Issues such as air and water purification, ozone based insecticides, and medical therapies using ozone, elicit a surprising range of responses from (1) "...vaccination offers no protection against disease"... instead, "... create ozone from pure oxygen and bring that into the body" (http://www.ozonio.com.br) to (2) "Ozone is bad news" (http://www.landmark.org/ozone.html) and (3) "Ozone has been proven to form...metabolites...thought to facilitate heart disease" (Scientific American.com, 31 May 2006; http://en.wikipedia.org/wiki/Ozone) to (4) "Down and dirty: Airborne ozone can alter forest soil" (http://www.innovations-report.com/html/reports/agricultural_sciences/report 22474.html).

Most interesting is the range of beliefs relating to ozone human therapy including venous ozone injection, ozone inhalation, and even rectal, otic, and vaginal insufflation for treatment of all manner of conditions including infection, cancer, and HIV. Although technically illegal in the US, since it is not approved by the FDA, at least 12 states have passed legislation allowing alternative therapies that include ozone and no less than 16 other nations, including France, Germany, Israel, Italy, and Russia allow medical ozone therapy on a regular basis. Not withstanding, at least one death has been reported in the US as a result of insufflation. Additionally, surface ozone has been determined to irritate the respiratory system and potentially harm lung function. Ozone has also been found to convert cholesterol in the bloodstream into plaque, hardening and narrowing arteries, and is also implicated in Alzheimer's disease (http://en.wikipedia.org/wiki/Ozone).

Ozone generated by corona discharge occurs when a feed gas, often air, containing oxygen is passed through a properly sized gap between a dielectric covered high voltage electrode and a ground electrode. When power is applied the electrical current generated excites the stable O_2 molecules forcing a portion to become reconfigured as ozone and resulting in a mixture of feed gas and ozone, the percentage of which determines the ozone concentration. Impurities in the feed gas may tend to reduce the ozone concentration as they compete to become reactants within the electrical field. The maximum ozone concentration produced by a generator that uses air as its feed is 50 g/m^3 and the maximum solubility concentration of ozone in ambient temperature water is approximately 40 mg/L (http://ewr.cee.vt.edu/environmental/teach/wtprimer/ozone/ozone.html). If the feed gas stream consists primarily of oxygen (O_2) then a higher concentration of ozone (O_3) molecules will be produced and a lesser amount of contaminants will typically interfere with ozone purity and volume. Inherent with the corona discharge process and in spite of the "Cold Generation" name, is the generation of heat which must be dissipated by some means, usually water or air cooling, since ozone production is severely restricted by elevated temperatures, especially 100°F and above. Additionally, the quality of the feed gas is critical not just from an overall purity perspective but also based on dryness. Feed gas quality will be discussed in more detail later on in this chapter. Dielectric life can be improved hence improving overall generator performance through the use of low relative operating voltages, (i.e., less than 4000 volts) and by avoidance of design or operation near the dielectric breakdown voltage (Figs. 2 and 3).

Alternatively, electrolytic ozone production uses pure water as the source for oxygen to convert to ozone eliminating the potential for contaminants common to gaseous feeds. In a typical electrolytic cell, the anode and cathode are separated by a solid polymeric membrane that serves as an electrolyte. A side stream of the process flow is connected to the anode side of the cell where some of the water is split into hydrogen and oxygen and then a portion of the oxygen is subsequently converted to ozone by electrical current. The developed ozone is immediately dissolved into the water which, along with any residual oxygen, is then reintegrated with the main process flow allowing the dissolved ozone to distribute throughout the process. "Waste" streams from

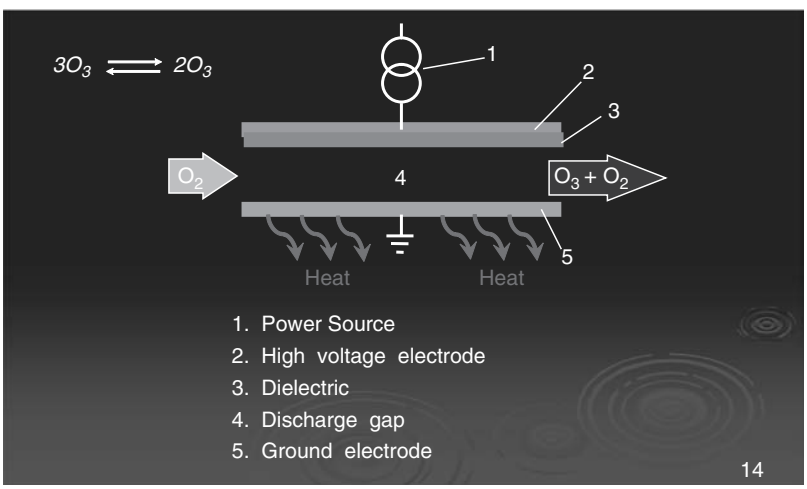


FIGURE 2 Corona discharge ozone generation. *Source*: Courtesy of Ozonia, NA.

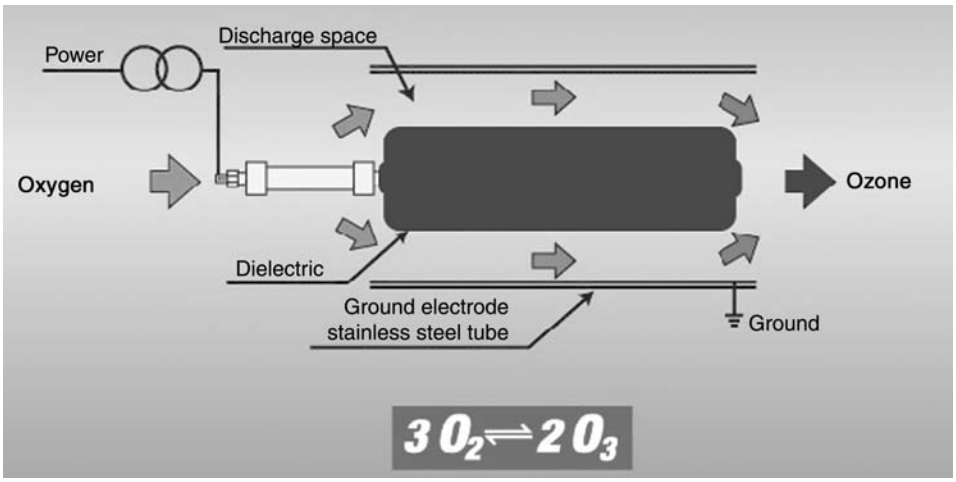


FIGURE 3 Tubular corona discharge O_3 generator. *Source*: Courtesy of Ozonia, NA.

the cathode side of the cell include a small amount of water and hydrogen residual from the disassociation of the water used to make the ozone. Since ozone is dissolved immediately in the process stream, undissolved ozone gas is not available for venting, mitigating the need for ambient ozone leak detection systems in most instances. The amount of ozone added to the process stream can be precisely controlled as the production rate, according to Faraday's Law, is proportional to the current flowing through the cell (Fig. 4).

Generally, the credit for the discovery of ozone is given to C.F. Schönbein, whose 1840 paper entitled "Research on the nature of the odor in certain chemical reactions" named ozone after the Greek word "ozein", meaning to smell. However, a Dutch chemist, Van Marum, was probably the first person to detect and note ozone's characteristic odor sensorially (in the vicinity of his electrifier) during his research (Lenntech, 2005). Other historical data relating to ozone is rather limited, although J.L. Soret is credited with determining the molecular structure of ozone, Werner von Siemens is credited with

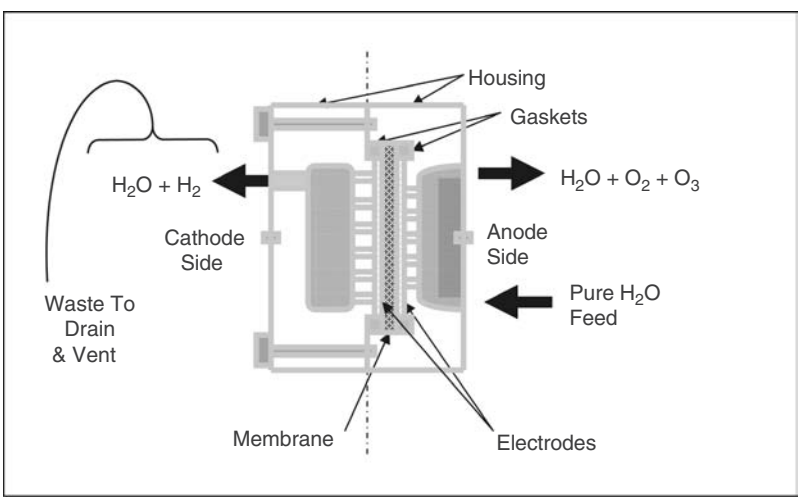


FIGURE 4 Electrolytic ozone cell. *Source*: Courtesy of Ozonia. NA.

development of the first ozone generator in 1857, and Marius Paul Otto appears to be the first to begin a specialized firm, Compagnie des Eaux et de l'Ozone, in France based on ozone as its primary product.

Apparently ozone's use as a disinfectant was recognized quickly, although it does not appear any one individual was primarily credited with that determination. Many studies followed von Siemens' invention with the first installation of ozone to disinfect drinking water at Oudshoorn in the Netherlands in 1893. Subsequently, ozonation of drinking water was implemented in Nice, France, in 1906, where it has continued without interruption for 100 years. Kramer and Leung note that during the same year New York City's 773,000,000 gallon Jerome Park Reservoir also began ozonation to control taste and odor, however it does not appear the practice was continued long term (Kramer, 2006).

Use of ozone grew in the early 1900s, however the post-WWI world saw it replaced, primarily by chlorine variants based on overall cost, ease of application, and higher yield. By 1940 there were only 119 ozonated drinking water applications worldwide and this figure had only grown to 1043 by 1977 with more than 50% of these located in France (Lenntech, 2005). In 1987, there were only five water treatment facilities using ozone in the United States. Not surprisingly, there has been a resurgence of interest in drinking water disinfection using ozone based on the discovery of THMs as a harmful disinfection byproduct of chlorine related sanitization.

PRACTICAL ISSUES RELATING TO THE USE OF OZONE

The competitive nature and resulting confidentiality within the healthcare industry has done little to allow for documented historical perspective relative to ozone usage, hence the following discussion is based on recognized industrial trends and reasonably available information, however, all site-specific installation data may not be included if it has been deemed to be of a proprietary nature, has remained undisclosed, or is unverified.

The application of ozone for sanitization of pharmaceutical water systems within the United States began in earnest during the 1980s with installations including both corona discharge and electrolytic technologies. Equipment available at that time was typically less than robust with the higher quality componentry available from Europe where the use of ozone was more prevalent. Implementation of ozone within a system creates interesting and unique challenges not just from ozone's application but based on traditional practices within the drug manufacturing arena.

Equipment necessary to generate ozone is only the starting point as the ozone must then be introduced into the process environment, monitored, controlled, and removed before use, all without impacting materials of construction, operator safety, patients, or final product quality and efficacy. Feed gas quality, temperature, ozone concentration, off-gassing, leakage, and other issues must be adequately addressed as well for a safe and effective installation. Although ozone application is not limited to process water, for the purposes of this discussion we will focus on that specific area. The reader is encouraged to review additional available material regarding; ozone sterilization of surgical suites, swimming pool sanitization, use in aquariums, direct human therapy, bottled beverages, drinking water, and the plethora of other applications available.

Initially, corona discharge generation was most prevalent based on its lower price and availability. Generators of that era were often constructed using a plate configuration for the ozone cell, with inherent flaws that manifest in frequent failures and resulted in substantial downtime, high maintenance costs, and leaks that posed potential injury to personnel.

Ozone monitors, for both gaseous ambient ozone as well as dissolved ozone, varied in their technologic approach to measurement, were expensive, unreliable, and difficult to maintain. Controls were unsophisticated and often capable of only on-off functionality compounding operational and reliability issues. Materials of construction and operational learning-curves resulted in a significant number of failures that compromised the ability of many plants to produce finished product while problems were being resolved. Off-gassing and ozone venting caused possible environmental concerns and leaks posed risks to personnel. Monitoring limits and other uncertainties created an added level of complexity that might have been expected to radically limit the roster of those willing to take on the added burden associated with implementation of an ozonated system.

Instead, the use of ozone has increased based on its efficacy and in spite of these noted problems, many of which have been fully or partially resolved. Today, systems incorporating ozone are considered reliable, cost effective, practical, and trusted for USP Purified Water production and for waters that serve as feed to Water for Injection systems. The FDA has given tacit approval for ozone application and many of ozone's vocal detractors have been silenced by the lack of operational problems and because the predictions of carcinogenic byproduct development have not come to fruition.

To fully understand the evolution that has taken place over the past two decades, it is prudent to track the changes and developments that have occurred; hence a brief review will follow discussing the improvements that have brought us to the current state-of-the-art. Details of each development including technological improvements are beyond the scope of this section, however materials listed in the bibliography can provide additional detail and support information.

GENERATION AND CONTROL

Many of the early generations of ozone equipment, especially corona discharge, utilized on-off control and employed printed circuit boards that were proprietary in design. The inability of this equipment to accept control input ultimately resulted in systems that exhibited erratic behavior and wide fluctuations in dissolved ozone levels. As a result, the technology was not viewed as robust or reliable, and systems were operated so that the lowest level of dissolved ozone that might be expected was still far above some arbitrary minimum selected as a baseline, with actual levels usually far above those needed to maintain control of organisms. These excessive levels commonly produced negative results including pH shifts and failures to meet quality standards. Excessive levels of dissolved ozone also resulted in accelerated attack of materials, especially gaskets, filter elements, and other less resistant materials. In addition, the previously noted design flaws compounded reliability issues as did the lack of ozone operating and maintenance experience. There were literally dozens of system failures from water entering the ozone generators based on poor injection design and poor piping techniques, both of which will be discussed further.

Minor pressure fluctuations caused plate type ozone generation cells to flex resulting in broken seals and leaks that jeopardized operators and systems alike. Vendors were marginally more experienced than users and not equipped to provide reasonable service response. As well, parts were seldom available from vendors stock increasing user frustration and cost. Feed gas, critical to ozone generator operation, was typically from either an existing plant compressed air supply or from a dedicated oxygen generator. During that period it was uncommon for either source to be monitored for quality and as a result any failures that occurred were typically attributed to the ozone generator.

However, once the impact of these systems was fully understood and monitoring systems installed, overall reliability began to steadily increase. It should be reemphasized that oxygen feed results in higher ozone concentration, reduced levels of undesired contaminants, and ultimately in lower operating costs. On-off control was exacerbated by demand issues such that tank storage levels and rate of fill often determined the amplitude of the dissolved curve and the amount by which levels overshot or undershot the desired setpoint.

Ozone monitoring and control instrumentation was also less than ideal during this period with both accuracy and reliability issues complicating the situation. Unfortunately this was the case for both dissolved and ambient monitors alike. Ambient monitors serve as leak detectors to protect personnel, however in one reported instance, the sample pump within the monitor failed with the monitor continuing to report "zero" levels of ambient ozone for almost two years before the problem was discovered. Obviously the user was primarily at fault, however the situation caused the manufacturer to reevaluate the unit design incorporating additional safety features in the future.

Although less likely to cause personnel injury, dissolved ozone monitors, including those manufactured in Europe where ozone use was more commonplace, also required modification to improve functionality. Problems with instrument reliability were caused by normal system pressure fluctuations or instrument drift resulting in out of calibration conditions far more frequently than predicted by the instrument manufacturer. Oftentimes, expensive pressure regulating valves are required to minimize the impact of pressure fluctuations even today, while many instruments require frequent recalibration to ensure they are consistently operating within specifications.

The prevalent technology for ozone monitoring incorporates electrochemical (EC) sensors and accounts for approximately 90% of installed applications. Alternatively, UV spectroscopic absorption units measuring either through the water matrix or by stripping the gas and measuring in the gas phase are also employed (Bloshine, 2006). Although UV sensors are excellent for high concentration gaseous measurement, they are not considered the best for dissolved ozone measurement primarily based on interferences and stripping complexities (Bloshine, 2006)

Electrochemical sensors rely on the polarographic principal and the cell consists of 2–3 electrodes, including an anode, a cathode, and possibly a guard-ring electrode submerged in an electrolyte, "A specific potential is applied to the cathode that will selectively reduce the gas analyte of interest" (Bloshine, 2006). The electrodes are covered by a gas-permeable membrane that will separate the gas from the liquid based on Henry's law, "which asserts that gases seek to be balanced across a permeable barrier" (Bloshine, 2006). Because the cathode chemically changes the gas, the effective concentration in the electrolyte always remains zero, thus keeping a constant permeation of gas into the cell. The current generated by the reduction of ozone at the cathode is proportional to the concentration of the ozone outside the cell, and adjusting for temperature and solubility yields a dissolved value (Bloshine, 2006).

As previously mentioned, accuracy of dissolved ozone measurement has been a recurring problem. This has most often been associated with off-gassing, as EC sensors cannot respond to mixed phase samples. Henry's law of partial pressures applies and off-gassing will occur when pressure fluctuations are present within the system as a result of equipment configuration and operational activity. Severe pressure gradients as well as pulsation and cavitation can even dislodge or damage (tear) the membrane resulting in erroneous readings and loss of process control.

Sensor locations within recirculated systems are important and worthy of mention. The primary sensor is normally located in the distribution piping after storage but before

ultraviolet decomposition, normally after the distribution pump(s). This sensor is linked to the ozone generator via the control system and modulates the generator output to maintain constant dissolved ozone levels based on setpoint. A second monitor is located immediately after the ozone destruct ultra-violet light. This monitor is used to ensure that ozone is not allowed to reach points-of-use during normal operation and may be interlocked with usepoints through the control system to restrict opening if ozone is present in the effluent. Conversely, this monitor can also be used to confirm that ozone is present, and its level, during sanitizations of the distribution system. Finally, a third monitor is also commonly included to measure and confirm proper return-loop ozone levels during sanitization as well as for back-up of the post-UV monitor.

Ambient ozone monitors are recommended for application in any areas where ozone gas leaks might pose a hazard to personnel, especially adjacent to the ozone generation system. In this regard it is common and considered good engineering to isolate the ozone generation system wherever practical and to include interlocks such that electrical power to the generator is disconnected and a room exhaust fan is activated if a leak occurs. As well, an illuminated warning light should caution personnel not to enter the containment if a leak is present, while also alerting appropriate individuals and creating an event log.

Calibration of ozone monitoring cells is an area of concern for metrology groups and operations personnel alike. There are two (2) methods for calibrating EC sensors; titration and air calibration. Both of these are indirect calibration methods as no bottled standards could be created and delivered based on ozone's half life. Blonshine (2006) mentions various titrants that are available to measure dissolved ozone including sodium arsenite, potassium iodine, and N,N-diethyl-p-phenylenediamine, however the International Ozone Association, the American Waterworks Association, and the US Environmental Protection Agency all recognize the indigo trisulfonate colorimetric method commonly called the "Indigo Method". The Indigo Method is relatively easy to perform but does require access to laboratory hardware and careful attention to detail including; sample temperature, ozone half-life, pH, water purity, exposure to light, and reference interferences (such as chlorine). Within these constraints, accuracies of ±10 ppb are achievable.

Air calibration, invented by Hale, is not only a reasonable alternative but in many cases a more accurate one. Hale recognized that oxygen could be measured with the same probe as used for ozone if the electrode potentials were changed and based on the many commonalities shared by ozone and oxygen. Accuracies of ± 5 ppb are possible making air calibration more accurate than titration. Unfortunately, even though air calibration has the added benefit of using no reagents, it can be problematic as the readjustment required from air back to dissolved ozone can take hours.

As a natural result of ozonation, undissolved gas, as well as gas liberated from the water requires venting which normally occurs from the storage vessel. When permitted by code, ozone may be discharged directly to the atmosphere where it reverts to oxygen (O_2) molecules relatively quickly. However, code notwithstanding, care must be taken not to discharge near building air intakes and windows, onto roofing materials or other unsuitable construction materials, or at points where impact to vegetation, animals or humans might occur. This type of venting, of necessity, typically occurs using a vent tube or pipe that discharges outside of a building however, protection of the vessel contents remains a concern.

Alternatively, when direct discharge is not allowed or when discharge within a building is necessary or desired, ozone must be removed to avoid damage to property or personnel injury. For these instances, ozone vent decomposers are typically installed. Vent decomposers of both the catalytic and thermal type are readily available. Catalytic units most often utilize manganese dioxide, which is used to speed the reaction of the

ozone back to oxygen. Units may also utilize fans to facilitate flow and many are equipped with heaters to reduce moisture that may cause operating problems. Moisture from the vent stream or from ambient outside air can cause the catalyst to solidify, mitigating its effect or worse, plugging the vent such that pressure or vacuum within the vessel causes operational issues, damage to equipment, or even injury. With vent decomposers typically mounted at the top of tall slender vessels, service was often difficult at best and ignored at worst, compounding a complex and possibly dangerous situation. Even pressure and vacuum rated vessels were not immune from problems as interior pressure and vacuum could; affect proper operation of pressure and level sensors, cause pump cavitation, and damage accessory equipment including the vent decomposer itself.

There are many options for introduction of ozone into the system from corona discharge generators, however many of the earliest were rife with problems. Some of these early designs utilized porous ceramic disks or "stones", similar to those used in fish tanks, to bubble the ozone gas into the bottom of the vessel. Bubbles were allowed to rise through the tall vertical column of water to enhance mass transfer, however these diffusers were contrary to sanitary design as they included threads, were not cleanable, and they were fragile with breakage causing reduction of dissolved ozone and downtime required for repairs. Other design difficulties included use of silicone gasketing (used because of the softness/low durameter) with a severely limited life in ozone contact and an extremely narrow application range (small tank diameter) since stones were only eight inches (8") in diameter (approximately), requiring multiple units for larger diameter tanks thus posing serious problems for balancing gas flow through each stone.

Subsequently, sparge tubes began to replace ceramic bubblers; however these only circumvented a portion of the issues relating to physical design but still required the ozone generator pressure to drive injection and the necessity for low point injection to assure intimate contact between the bubbles and the bulk water. Current designs utilize a venturi eductor system that minimizes the need for the ozone generator to overcome line pressure, and is often coupled with an in-line static mixer to ensure suitable ozone distribution.

These advancements result in higher levels of dissolved ozone, lower ozone production, and more even gas distribution at minimal additional cost and while reducing the likelihood water will flood the ozone cell causing damage to the generator. As a result, vessels are no longer required to be tall and thin to aid ozone contact time offering more flexible installation options and system configurations, including the use of horizontal vessels. When "bubbling" is necessary for the dissolution of ozone, basic mass transfer rules are applicable such that smaller bubbles result in greater exposed surface area and better transfer characteristics. High quality "stones" are capable of bubble sizes of about 30 micron.

It should be noted that in spite of improvements in ozone injection and in generator design it remains an appropriate design practice to ensure the ozone feed line to the vessel is routed in a fashion such that it is higher than the water level in the vessel. This will assure that in a system-wide failure, water will not flood the generator causing additional damage.

As previously cautioned, ozone venting must be addressed, however this issue is complicated by the necessity to protect the vessel contents from microbial contamination. Under dynamic conditions, water will continually fill and empty from the system. This situation will result in tank level fluctuation including air in-rush during emptying and combined air and ozone venting during filling, unless nitrogen blanketing is installed. Air

entering the vessel may contain particulate as well as bacteria that could compromise the water quality and as a result, a microbially retentive filter must be included in the design scheme. For permitted applications a vent filter, typically rated at 0.2 micron absolute retention can be placed in the vent line to clean the air entering the vessel. This unit must be compatible with ozone gas, must be hydrophobic to avoid plugging from moisture, and may require a heating jacket as well as other accessories such as vent and drain valves to ensure proper operation and testing. Alternatively, when used with a vent decomposer, the vent filter should be placed below the vent decomposer to perform the tasks mentioned above and to minimize the potential for particulate, catalyst, and other foreign matter from the decomposer to enter the tank including any resident microbes. The added benefit of this arrangement is that the sanitary vent filter serves as a barrier between the non-sanitary vent decomposer (as of this writing, sanitary designed vent decomposers have yet to become available) and all other portions of the sanitary system. It would be prudent to mention that numerous system failures have occurred over the years as a result of ozone vent decomposer failures including microbial contamination, collapsed tanks, and catalyst resin suspended in product water distributed to use-points throughout the facility.

Unfortunately, vent filter elements have been typically constructed of plastic materials including polypropylene that have limited functional life in ozone environments. This limitation necessitates frequent testing and change-out at significant cost since no better options are available. Sintered metal filters, rated at 0.45 micron in a gas stream and 1 micron in a liquid stream (approximately) are considered inadequate although their stainless steel construction can last indefinitely under even the harshest ozone concentrations. There has also been substantial discussion regarding filter elements constructed entirely of Teflon^{TM}, including both the media and support structure, however this has yet to come to fruition at a commercial level.

In can be surmised that the market for ozone resistant vent filter elements may not be large enough to justify a significant investment in research and development to bring a suitable product to the marketplace and there is little added motivation on the part of vendors who will likely see a reduction in sales if this were to occur. Coupled with the tendency by pharmaceutical companies to maintain the status quo, little change is expected as a result of healthcare industry initiative.

For drinking water applications, additional concerns such as those relating to fertilizers, herbicides, and pesticides are prevalent. Atrazine and other triazines merit special mention as they are difficult to remove. However the addition of hydrogen peroxide to ozone ($O_3 + H_2O_2$) produces hydroxyl radicals effective in the destruction of these undesired compounds. As well, the combination of ozone and hydrogen peroxide can be effective in elimination of unpleasant tastes and odors. Although the free radical (HO°) produced from the combination of ozone and hydrogen peroxide is effective in removing trace organics, it must be noted that this activity usually leaves no residual ozone remaining for downstream disinfection, a situation that obviously cannot be overlooked.

For almost five decades prechlorination of raw water was the method of choice to prevent virtually all biologic activity, primarily based on cost and also because of our ignorance relating to the formation and impact of THMs. Today, preozonation has become fairly common in European drinking water pretreatment as cost differences have become less substantial and in light of the discoveries relating to the disadvantages of chlorination byproducts. Preozonation of raw water as part of drinking water preparation is however significantly different than preozonation in a pharmaceutical water system since it would be expected that feedwater to most pharmaceutical water systems contains chlorine, in at least minimum amounts.

Most public suppliers of potable water view their constituency primarily as residential users who are uneducated and unconcerned, who trust that they are being protected, and who require little information about the methods being employed. As a result, when treatment technology is modified, as in the change from chlorine to chloramines, there is little fanfare or publicity. For residential users these changes seldom have significant impact and are most often overlooked. However, on the commercial and industrial side, these changes can have tremendous effect ranging from equipment sizing to materials of construction issues. As an example, granular activated carbon (GAC) filters, used for chlorine and organics removal, may be undersized for chloramine removal if they were originally sized for normal and expected levels of chlorine. Situations of this type have occurred often over the past few years as suppliers of potable water convert to chloramine without notice to users, affecting systems designed and installed 1–10 years ago based on then current data and technology. Even worse, newer systems may utilize ultraviolet for chlorine removal to protect RO membranes and these too may be undersized if a change to chloramines is surreptitiously implemented, irreversibly damaging expensive RO membranes and causing system outages with associated lost time and ruined product.

Additionally, changes such as to chloramines can have other surprising affects on system performance and materials. In two documented cases, systems originally designed with stainless steel pretreatment hardware, residual chlorine from the treatment source, and in-plant preozonation resulted in component failures within a short period after conversion to chloramines. The failure mode was accelerated chloride attack of the stainless steel as confirmed by an independent laboratory. Both systems had operated for many years without failure in spite of the combined chlorine and ozone. Yet, within very short periods (in one case, as little as 48 h) after conversion to chloramines, stainless steel components failed catastrophically exhibiting classic signs of chloride based crevice corrosion. Since the simple conversion to chloramine has not induced failure in other stainless systems and since the combination of chlorine and ozone had not resulted in previous failures, it has been deduced that the replacement of chlorine by chloramine in an ozonated environment resulted in accelerated attack and the noted failures. Although significant further study did not occur, repeated short term failures of individual components served to confirm the assertion that the combination of ozone and chloramine led to accelerated metallic attack, possibly enhanced by other contributing but undetected factors.

Ozone's use as an organic oxidant is primarily based on the cleavage of the carbon double bond which acts as a nucleophile or a specie having excess electrons (Aeppli and Dyer-Smith, 1996). As a result, ozone will always react directly with organic compounds

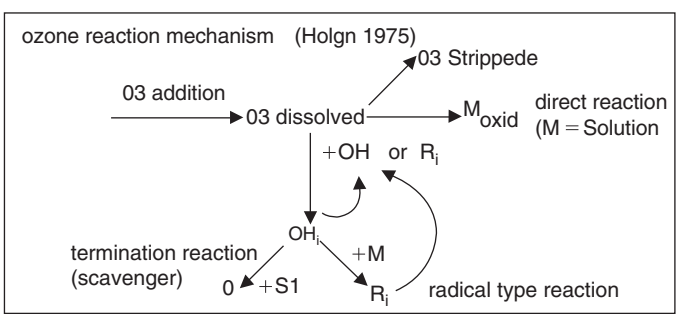


FIGURE 5 Ozone reaction mechanism (hoigné). *Source*: From Aeppli and Dyer-Smith (1996).

$$\frac{d[O_3]}{dt} = k_{o3}[M] \bullet [O_3]_t$$

Where:
k_{o3}: reaction rate constant (l.mole^{-1} .s^{-1})
M: solute concentration (mole.l^{-1})
O_3: ozone concentration (mole.l^{-1})

FIGURE 6 Ozone consumption reaction. *Source*: From Aeppli and Dyer-Smith (1996).

and also by reaction of free hydroxyl radicals. This dual reaction model was described by Hoigné in 1975 and is reproduced below for clarification (Fig. 5).

As well, Aeppli and Dyer-Smith noted that "the consumption of ozone by direct reaction can be written (below) as a pseudo first order reaction if the solute concentration is higher than the ozone concentration" (Aeppli and Dyer-Smith, 1996) (Fig. 6).

It should be noted that direct reaction is heavily dependent upon the organic compound's chemical nature, while the reaction of the radical is primarily independent although a few orders higher than those of the direct reaction (Aeppli and Dyer-Smith, 1996). Reaction rate constants for various ozone and hydroxyl radical reactions with organic compounds are listed below in Table 3.

Aeppli and Dyer-Smith were also careful to note that in the reaction chain, species of aldehydes or ketones appeared twice as reaction products and the carboxylic acids only once as further oxidation of these products, based on their low reaction rate constants, will not easily occur. As well, aldehydes and ketones are known nutrients for bacteria making organic loading an important consideration when considering ozone for preozonation. This is obviously less of concern after purification, especially for pharmaceutical applications with TOC limitations, however it should not be ignored completely as it cannot be considered insignificant.

The application of corona discharge for ozone production requires careful consideration of the feed gas quality as it is the most critical aspect involved in creating pure gaseous ozone that is not laden with contaminants, moisture, or reactants, any of which can hamper the effectiveness, ultimately reduce the degree of microbial kill, and potentially contaminate the products it contacts.

TABLE 3 Reaction Rate Constants K of Ozone and Hydroxyl Radicals with Organic Compounds

Compound	K (L.mole^{-1}.s^{-1})	°OH
Olefins	1000–4.5×10^5	10^9–10^{11}
S-organics	10–1.6×10^3	10^9–10^{10}
Phenols	10^3	10^9
N-organics	10–10^2	10^8–10^{10}
Aromatics	1–10^2	10^8–10^{10}
Acetylenes	50	10^8–10^9
Aldehydes	10	10^9
Ketones	1	10^8–10^9
Alcohols	10^{-2}–1	10^9–10^{10}
Alkanes	10^{-2}	10^6–10^9

Source: From Aeppli and Dyer-Smith (1996).

It can be appropriate to use either air or oxygen to produce ozone, however for high purity applications, including pharmaceuticals, oxygen rich (90^{+}%) feed gas is highly recommended by equipment manufacturers. It is also recommended that the feed gas be; clean and particle free above 0.4 μm, dry to −60°C dew point or lower, and oil and hydrocarbon free. It is also extremely important that the feed gas be plumbed using appropriate materials of construction to avoid contamination, especially particulate that can either react with ozone, compromise the process, or both. With lower levels of impurities in the feed gas higher concentrations (wt.%) of ozone can be achieved, and as a result, increased gas absorption into the water is possible resulting in more effective disinfection, lower operating cost, and possibly even lower costs for capital equipment based on physical size and purchase expense. Although lower concentrations of ozone are common, in the range of 5%, higher concentrations, in the range of 10–12% by weight are considered optimal. As an example, note the difference in oxygen (O_2) required to produce 10 pounds of O_3 at 5% concentration (200#) versus that required at 12% concentration (83#).

Appropriately sized plant compressed air systems that meet the purity requirements noted above can be used to produce ozone, however, many facilities chose an alternate route since upgrading and maintaining a large diverse system simply to accommodate a single user may prove cost prohibitive. Plant compressed air systems are seldom categorized critical direct contact systems although that status would most likely change if ozone production were added to the list of uses. Therefore, it is not uncommon for system design to include a dedicated source of oxygen that has been earmarked solely for supply of oxygen to the ozone generation system. One possible method of providing oxygen for the ozone system is from the vaporization of liquid oxygen (LOX) stored on-site in cylinders or in a bulk storage vessel. This can become expensive and can also result in outages if refilling is delayed. Furthermore, reliance on a vendor for delivery combined with the requirements for vendor qualification and subsequent auditing can substantially increase the per-unit cost making this option less desirable.

Alternatively, oxygen generation equipment can be sized and selected solely to supply oxygen to the ozone generation system. Two primary types of oxygen generators are produced and they are designated as pressure swing adsorption (PSA) type and vacuum swing adsorption (VSA) type generators. For smaller applications, and most pharmaceutical applications fall into this category, PSA generation is most practical. Pressure swing adsorption technology involves the passage of compressed (30–90 psig) air through a vessel containing molecular sieve material. The sieve, having a greater affinity for nitrogen and other gasses, including moisture, retains all but the oxygen and about 4% argon. Prior to becoming fully saturated, the sieve, is regenerated by depressurization (desorption) followed by an oxygen purge. The majority of PSA generators utilize at least two (2) pressure vessels, also known as adsorbers or beds, to allow one vessel to be regenerated while the other is in service. An oxygen receiver tank connected to the outlet of the system stores the 90–95% pure oxygen at constant pressure to eliminate fluctuations and possible downtime. The product oxygen is typically produced between 5–60 psig, has a dew point of approximately -100° Fahrenheit and is 90–95% pure.

Vacuum swing adsorption, also known as vacuum pressure swing adsorption (VPSA) is similar to PSA however, low pressure, high volume blowers are utilized for the adsorption and vacuum blowers are used for desorption. Reduced air inlet pressure translates to lower oxygen output pressure, most commonly in the range of 3–5 psig unless an oxygen booster or compressor is added. Oxygen produced is in the range of 88–94% pure and a dew point at -100°F parallels that from the PSA generator. Both PSA and VSA/VPSA employ the same 3:1 ratio of adsorption pressure to regeneration

pressure, although the regeneration pressure for VSA is below atmospheric. VSA also commonly employs dual adsorbers and can be well suited to large volume applications where ambient air is simply drawn into the pressure blower with minimal prefiltration.

For large applications, such as 10,000 to 80,000 SCFH, VSA/VPSA is the more practical alternative and when lower purity and pressure is acceptable VSA is generally in the range of 30% more energy efficient. Pressure swing adsorption oxygen generators are typically fed from a plant compressed air source and are less expensive on a first-cost basis. In addition, they are also more compact, quieter, more reliable, and less maintenance intensive than the VSA/VPSA alternative, providing feed gas temperature is below 122°F.

As mentioned previously, ozone can be formed by electrical discharge such as lightning but also from high energy electromagnetic radiation. In addition, electrical equipment can generate significant amounts of ozone when high voltages are employed such as is common in laser printers, photocopiers, electric motors, and welding systems. This has given rise to concerns for employee safety in the workplace not just for those operating and maintaining heavy equipment but also for office employees surrounded by computer related electronic equipment resident in their workspace.

SUMMARY

Ozone is an effective sanitizing agent appropriate for use in pharmaceutical, biotech and other healthcare related applications. Ozone is especially suited for microbial control in water systems including those that must comply with monographs promulgated within the US Pharmacopeia and also with FDA requirements. Based on its oxidative strength, ozone is extremely useful but poses certain challenges including selection and maintenance of proper materials of construction and suitable design for the protection of personnel and products. There is a continually expanding body of knowledge relating to ozone's application and use which must be clearly understood if ozone's full benefit is to be derived.

Ozone has the capacity to offer reliable, cost effective treatment for microbial control and as expenditures for pharmaceutical and healthcare products continue to rise, traditional technologies will experience increased scrutiny, and ozone will most likely continue to gain in popularity with greatly expanded use and further development of applications and technical information.

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about the book...

Filtration and Purification in the Biopharmaceutical Industry, Second Edition greatly expands its focus with extensive new material on the critical role of purification and the significant advances in filtration science and technology. This new edition provides state-of-the-science information on all aspects of filtration and purification, including the current methods, processes, technologies and equipment, and brings you up-to-date with the latest industry standards and regulatory requirements for both the pharmaceutical and biopharmaceutical industries.

An essential, comprehensive, source for all professionals involved with filtration and purification practices and compliance, this text:

- addresses recent biotechnology-related processes and advanced technologies, such as viral retentive filters, membrane chromatography, downstream processing, cell harvesting, and medium and buffer filtration
- presents detailed updates on the latest FDA and EMEA regulatory requirements involving filtration and purification practices
- describes current industry quality standards and validation requirements and provides guidance for compliance

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